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UNIVERSITY OF ALBERTA

MODULATION OF CALCIUM CHANNELS AND MEDIATION OF THE α_1-ADRENOCEPTOR EFFECT IN RAT MYOCYTES: ROLE OF G PROTEINS

BY

QIN-YUE LIU ©

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

DEPARTMENT OF PHYSIOLOGY

EDMONTON, ALBERTA

SPRING, 1993

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Modulation of Calcium Channels and Mediation of the α_1-Adrenoceptor Effect in Rat Myocytes: Role of G Proteins" submitted by Qin-yue Liu in partial fulfillment of the requirement for the degree of Doctor of Philosophy

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DEDICATION

To

My parents
Tianpei Liu & Menren Rao,
eternal scholars

ABSTRACT

G proteins are known to modulate voltage-dependent Ca^{2+} channels. In different cell types, G proteins may have distinct effects on voltage-dependent Ca^{2+} channels. Thus, four types of cells which represent various biological tissues were chosen to investigate the effect of GTPγS, a G protein activator, on voltage-dependent Ca^{2+} channels. In this thesis, L- and T-type Ca^{2+} channels were studied using the whole cell version of the patch clamp technique. The intracellular application of GTPγS initially increased Ca^{2+} currents and then decreased them in GH_3 cells (endocrine cell), GTPγS increased Ca^{2+} currents in dissociated smooth muscle cells from rat tail artery (VSMC) and neonatal rat ventricular cells (cardiac myocytes), but did not affect T current in N1E-115 cells (peripheral neurones). Application of two toxins to GH_3 cells demonstrated that a PTX-sensitive G protein is responsible for the initial increase and a CTX-sensitive G protein is involved in the subsequent decrease in Ca^{2+} current.

Since GTPγS activates all the G proteins in a given cell, its effects on Ca^{2+} currents result from the summation of all activated G proteins. In order to further characterize a specific G protein involved in the modulation of Ca^{2+} channels, one type of cell (myocytes) was used to study the G protein pathway from receptor (α_1-adrenoceptor) to effector (L-type Ca^{2+} channel). Under physiological conditions, G proteins couple receptors and effectors. Phenylephrine, an α_1-adrenergic agonist, increased L-type Ca^{2+} channel current in neonatal rat ventricular cells. However, this effect was not observed in adult rat ventricular cells. The effect of phenylephrine on L current is concentration-dependent and acts exclusively through α_1-adrenoceptors. A PTX- and

CTX-insensitive G protein and PKC are responsible for mediation of the phenylephrine-induced increase in L current.

This is the first demonstration that stimulation of α_1-adrenoceptors in neonatal but not adult rat heart induces an increase in the L-type Ca^{2+} channel current. This mechanism is mediated by a PTX- and CTX-insensitive G protein and PKC.

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LIST OF ABBREVIATIONS

AC	adenylate cyclase
ACh	acetylcholine
BK	bradykinin
BSA	bovine serum albumin
$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
caged-GTPγS	guanine-5'-O-(3-thiotriphosphate),3-s-[1-(2-nitrophenyl)ethyl]thioester
cAMP	adenosine 3',5'-cyclic monophosphate
CTX	cholera toxin
DG	diacyglycerol
DRG	dorsal root ganglion
DHP	dihydropyridine
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethylsulfoxide
EGTA	ethyleneglycol-bis-(β-aminoethyl ether)N,N,N',N'-tetraacetic acid
4-AP	4-aminopyridine
FBS	fetal bovine serum
FCS	fetal calf serum
FD	fast deactivation calcium channel
HBSS	Hank's balanced salt solution

HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HVA	high voltage activated calcium channel
GDPβS	guanine-5'-O-(2-thiodiphosphate)
GH_3 cell	pituitary adenoma cell line
Gpp(NH)p	guanylyl-imidodiphosphate
G protein	guanine nucleotide binding protein
GTPγS	guanosine-5'-O-(3-thiotriphosphate)
I_{Ca}	calcium current
I_{K+}	inwardly rectifying background potassium current
IP_3	inositol 1,4,5-triphosphate
I_{Si}	slow inward current
I_t	transient outward potassium current
I-V relationship	current voltage relartionship
LHRH	luteinizing hormone releasing hormone
LTs	leukotrienes
L-type Ca^{2+} channel	long-lasting calcium channel
LVA	low voltage activated calcium channel
MOPS	3-[N-morpholino]propanesulfonic acid
N1E-115 cell	undifferentiated murine neuroblastoma cell line
NMDA	N-methyl-O-aspartate receptor
NPY	neuropeptide Y
OAG	1-oleoyl-2-acetyl-rac-glycero

pCa	-log[Ca^{2+}]
PGs	prostaglandins
PI	phosphatidylinositol
PIE	positive inotropic response
PIP_2	phosphatidylinositol 4,5-bisphosphate, PI-4,5-P_2
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PNS	penicillin, neomycin and streptomycin
PMA	4β-phorbol 12-myristate 13-acetate
PTX	pertussis toxin
R_p-cAMPs	adenosine 3',5'-cyclic monophosphothioate R_p-isomer
SD	slow deactivation calcium channel
S_p-cAMPs	adenosine 3',5'-cyclic monophosphothioate S_p-isomer
SR	sarcoplasmic reticulum
T-type Ca^{2+} channel	transient type calcium channel
TTX	tetrodotoxin
VDCC	voltage-dependent Ca^{2+} channel
VSMC	vascular smooth muscle cell

CHAPTER I. INTRODUCTION AND LITERATURE REVIEW

The concentration of cytosolic free calcium ($[Ca^{2+}]_i$) is important for the control of many essential cellular activities. Typically, the $[Ca^{2+}]_i$ of quiescent cells is 50-200 nM. The Ca^{2+} concentration of the extracellular fluid is approximately 1-2 mM. Hence, there is a large concentration gradient of Ca^{2+} across the cell membrane. Electrical or chemical stimulation of the cell will increase the $[Ca^{2+}]_i$ which in part is due to Ca^{2+} influx across the cell membrane. This influx is due to the movement of Ca^{2+} down its concentration gradient through membrane pores which are selective for this ion. These pores are part of the more complex structures known as Ca^{2+} channels. These channels consists of two basic classes, ligand-gated calcium channels (receptor-operated) and time- and voltage-dependent calcium channels (VDCC). The ligand-gated Ca^{2+} channels require ligand and receptor interaction for activation. The activation of VDCC requires depolarization of the cell membrane. Voltage-dependent Ca^{2+} channels can, however, be modulated by hormones, neurotransmitters and drugs (Reuter, 1983). Most hormones and neurotransmitters modulate VDCC through transmembrane transducers which are guanine nucleotide binding proteins (G proteins). Hence, G proteins play an obligatory role in the modulation of VDCC by hormones and neurotransmitters. The hypothesis which forms the basis of this thesis is: G proteins play a central role in the modulation of voltage-dependent Ca^{2+} channels by various extracellular agents. G protein modulation of Ca^{2+} channel currents was studied using the whole cell version of the patch clamp technique.

Various G proteins are present in different cell membranes and they are associated with distinct channel proteins. Therefore, G protein activation could decrease, increase or have no effect on channels depending on the cell. The first part of this study is focused on the involvement of G proteins in the modulation of VDCC in different cell types. The VDCC which are studied in this thesis are the long-lasting (L) and the transient (T) Ca^{2+} channels. Four types of cells were chosen for this study. The GH_3 cell line (a clonal pituitary tumour cell) represents an endocrine secretory cell. N1E-115, a neuroblastoma cell, represents neuronal tissue (peripheral neurons). Smooth muscle cells from rat tail artery represent the vascular smooth muscle cell (VSMC) and neonatal rat ventricular cells represent ventricular myocytes. In GH_3 cells, rat tail artery smooth muscle cells and ventricular myocytes, both T- and L-type Ca^{2+} channel currents can be measured (Armstrong & Matteson 1985; Wang *et al.*, 1989; Liu *et al.*, 1992a). If N1E-115 cells are maintained under determined culture conditions (Wang *et al.*, 1990), they express primarily T channels (Liu *et al.*, 1992b). The effect of guanosine-5'-0-(3-thiotriphosphate) (GTPγS) on the voltage-dependent Ca^{2+} channel currents in four types of cells was investigated. This study provides fundamental evidence to support the above hypothesis and extends it to four cells and some of the VDCC associated with these cells. Although it is widely accepted that G proteins modulate VDCC in various tissues, this is the first comparison of the effect of GTPγS on VDCC in four different tissues under the same experimental conditions.

G proteins are a family of proteins with many subtypes (Birnbaumer, 1990). GTP and its analogues have the ability to modify whole cell calcium channel currents in the absence of agonists (Dolphin *et al.*, 1988). GTPγS is a non-specific activator. It stimulates all G proteins. The results produced by GTPγS are the summation of the effect of all the activated G proteins. For example, if two or more G proteins are coupled to Ca^{2+} channels in a particular cell, the effect of GTPγS on these Ca^{2+} channel currents would be the summation of the effects of the individual G proteins. It is known that under normal physiological conditions, G proteins play a central role connecting the receptor to the effector. In order to study a specific case where a receptor is coupled to a G protein which, in turn, modulates a specific voltage-dependent Ca^{2+} channel, the α_1-adrenoceptor in neonatal rat myocytes was selected. This constitutes the second part of this thesis. The α_1-adrenergic response in the neonatal heart has been reported to be modulated by a pertussis toxin (PTX)-insensitive G protein (Steinberg *et al.*, 1985). This G protein caused phosphatidyl inositol 4,5-bisphosphate (PIP_2) hydrolysis to produce 1,4,5-inositol triphosphate (IP_3) and diacylglycerol (DG) in response to α_1-adrenergic stimulation (Steinberg *et al.*, 1987, 1989). In studies of ventricular tension, it has been reported that the inotropic response to α_1-adrenergic stimulation has two phases: a transient and a delayed sustained phase. IP_3 was found to be associated with the transient phase and protein kinase C (PKC) with the sustained phase (Otani *et al.*, 1988; Talosi & Kranias, 1992). PKC itself has been demonstrated to increase the Ca^{2+} channel current in neonatal rat ventricular cells (Dosemeci *et al.*, 1988; Lacerda *et al.*, 1988). Based on this information, it is possible that a PTX-insensitive G protein may functionally couple the α_1-adrenoceptor to the VDCC. The stimulation of the α_1-adrenoceptor causes the activation of a PTX-insensitive G protein which results in PKC activation and an increase in Ca^{2+} current resulting from phosphorylation of channel proteins. The α_1-adrenoceptors in the heart are well documented in terms of the receptor characterization and their effects, i.e., the inotropism and chronotropism in the intact heart. However, the α_1-adrenergic response in the heart is poorly characterized with respect to its iontropic mechanisms. Therefore, an α_1-adrenergic agent, phenylephrine, was used in this thesis to map out the pathway: α_1-adrenoceptor---G protein---second messengers---Ca^{2+} channel---inotropic response. This is the first report to describe a pathway for the inotropic response due to the α_1-adrenergic stimulation in neonatal rat heart. However, some other pathways may also be involved in the inotropic response caused by α_1-adrenoceptor activation. For example, α_1-adrenoceptor activation may increase Na^+/K^+ pump rate (Shah *et al.*, 1988) or they may alkalinize the cell interior and increase the sensitivity of myofilaments to Ca^{2+} (Terzic *et al.*, 1992).

The α_1-adrenoceptor pathway described above has been reported to be different in the adult rat and, hence, in the last part of this work, the results from neonates were compared to the results from adult rats. The literature review which follows covers the diverse fields which this study attempts to address.

1. G Proteins and Calcium Channels

1.1. G Proteins

1.1.1. *G Proteins and Their Activation*

In view of the importance of the control of cellular functions by changes in $[Ca^{2+}]_i$, it is of interest to explore the mechanisms by which Ca^{2+} fluxes through cell membranes are regulated. On the basis of current knowledge, it is believed that a family of G proteins serves as membrane-bound transducers. They modulate calcium channels in a number of cells (Williamson, 1986; Litosch, 1987; Spiegel, 1987). The G protein family derives its name from the specific use of GTP by its members. These proteins contain three subunits (designated α, β and γ) which interact directly with receptors on the surface of the cell. The α-subunit contains a single, high-affinity binding site for guanine nucleotides and possesses the GTPase activity that is crucial for the action of these proteins. Differences in the α subunit serve to distinguish the various G protein oligomers. At present, the G protein family is known to contain at least sixteen different genes that encode the α subunit of the heterotrimer, four that encode β subunits and multiple genes encoding γ subunits (Simon *et al.*, 1990). Functionally, G proteins can be divided into several subtypes. G_s is the stimulatory G protein for adenylate cyclase, G_i is the inhibitory G protein for adenylate cyclase, G_o is the other G protein with unknown functions, G_q is the pertussis toxin- and cholera toxin-insensitive G protein for PIP_2 hydrolysis (Smrcka *et al.*, 1991), and transducin is the G protein in rod and cone cells of the retina (Gilman, 1987). There are multiple forms of the subunit polypeptides within each subtype. The existence of two forms of $G_{s\alpha}$ (α subunit) was established when the protein was purified. According to their molecular weights, $G_{s\alpha}$ is classified as $G_{s\alpha 45}$ and $G_{s\alpha 52}$. The 45 and 52 KDa forms of the α chain arise from alternative splicing of a primary transcript encoded by a single gene. G_i contains an α subunit of 40-41 KDa that is a substrate for ADP-ribosylation by pertussis toxin. Three highly homologous cDNAs that encode putative $G_{i\alpha}$ subunits ($G_{i\alpha 1}$, $G_{i\alpha 2}$ and $G_{i\alpha 3}$) have also been isolated (Casey & Gilman, 1988).

Activation of G proteins involves an exchange of guanine nucleotides and an apparent dissociation of the α-subunit from the $\beta\gamma$-subunits. A G protein in the basal state has bound GDP and exists primarily as a trimeric oligomer. During activation by a receptor, GDP is released and replaced with GTP. The α-subunit, when bound to GTP, has a lower affinity for $\beta\gamma$ and the activated G protein probably exists primarily as the dissociated α-GTP and $\beta\gamma$-subunits. It is apparent that two potentially regulatory molecules are produced. The activated α-GTP activates various effectors, i.e., enzymes. The α-subunits also contain intrinsic GTPase activity which is involved in the inactivation of the reaction. The activated α-GTP is converted at a relatively slow rate (4-10 GTP molecule/G protein/min *in vitro*) to α-GDP, which then reassociates with the $\beta\gamma$-subunits to complete the cycle (Sternweis & Pang, 1990). GTPγS, a non-hydrolysable GTP analogue, and GDPβS, a non-hydrolysable GDP analogue, are often used to investigate the involvement of G proteins in the modulation of cellular events.

1.1.2. *Toxins*

The α-subunits of the G proteins also contain the sites for NAD-dependent ADP-ribosylation which can be catalyzed by bacterial toxins. The α subunit of G_s ($G_{s\alpha}$) and G_t ($G_{t\alpha}$, transducin) can be ADP-ribosylated by cholera toxin (CTX) and the polypeptides currently designated as $G_{i\alpha}$ and the similar $G_{o\alpha}$ can be ADP-ribosylated by pertussis toxin

(PTX). Modification of G_s or G_t by cholera toxin increases their activity while modification of G_o, G_i and G_t by pertussis toxin reduces their activity (Ui, 1984; Gilman, 1987). Therefore, pertussis toxin and cholera toxin are widely used as unique and valuable probes for G proteins. With the aid of these bacterial exotoxins, not only can the effect of G proteins be verified, but also certain types of G proteins can be identified.

1.2. **Calcium Channels**

Current knowledge regarding Ca^{2+} channels has dramatically increased due to patch clamp and other electrophysiological studies during the past decade. The structures through which Ca^{2+} permeates are "pore-" or "channel-" forming proteins embedded in the lipid bilayer of the membrane. Ca^{2+} channels are usually classified into two basic groups, voltage-dependent Ca^{2+} channels (VDCC) and ligand-gated Ca^{2+} channels (receptor-operated). The classification was based on functional grounds and has now been confirmed by the amino acid sequence similarities of members of each group (Hille, 1989).

1.2.1. ***Time- and Voltage-Dependent Calcium Channels (VDCC)***

Time and voltage-dependent Ca^{2+} channels are the most extensively studied class. They include Ca^{2+} channels activated by rapid depolarization and also modulated by neurotransmitters and hormones (Tsien & Tsien, 1990). Neurotransmitters and hormones can modulate VDCC through G proteins and/or second messengers. Direct activation of

G proteins (GTPγS) in the absence of ligands can also modulate VDCC directly or indirectly through second messengers. Intracellular second messenger modulation of VDCC is mainly driven by a ligand-receptor interaction and G protein activation.

(A) *Depolarization-Activated Ca^{2+} Channels*

The voltage- and time-dependent properties of these Ca^{2+} channels clearly distinguish them from pumps or exchange mechanisms (Tsien, 1983). The depolarization-activated Ca^{2+} channels can be classified into various subtypes (Hagiwara & Byerly, 1981) according to their gating, ionic conductance, pharmacology and cellular distribution (Tsien *et al.*, 1988). The co-existence of multiple types of Ca^{2+} channels within a cell has been demonstrated in many cells using voltage clamp experiments (Tsien *et al.*, 1988). The most extensively used classification categorizes VDCC as L, T, N, and P Ca^{2+} channels (Tsien & Tsien, 1990). In some types of cells, i.e., vascular smooth muscle cells, it has been suggested that this classification is an oversimplification (Bolton *et al.*, 1988).

(i) L-Type Ca^{2+} Channels

The Ca^{2+} channels which inactivate slowly (long-lasting) and require high voltage to activate (HVA) are usually classified as the L-type Ca^{2+} channels. The L-type Ca^{2+} channels are sensitive to 1,4-dihydropyridines (DHPs). The single channel conductance of the L-type Ca^{2+} channel is 25 pS (McCleskey *et al.*, 1986). This channel is ubiquitous in excitable cells and many non-excitable cells. It is the major pathway for voltage-gated

Ca^{2+} entry into cells (Tsien *et al.*, 1990). L-type Ca^{2+} channels are involved in the activation of contraction in the heart and most kinds of smooth muscle and in the control of transmitter release from neurons and in secretion from endocrine cells. They are the main target of Ca^{2+} antagonist drugs such as verapamil, diltiazem or DHPs. These channels are also affected by endogenous compounds (Janis *et al.*, 1988; Callewaert *et al.*, 1989). In many cells, the L-type Ca^{2+} channels are strongly modulated by catecholamines, which usually act via cAMP-dependent protein phosphorylation (Kameyama *et al.*, 1985; Tsien *et al.*, 1986; Yue & Marban, 1990). There is also evidence that catecholamines gate L-type channels directly through the G protein (G_s) (Yatani & Brown, 1989).

(ii) T-Type Ca^{2+} Channels

T-type channels are sometimes called low-voltage activated (LVA) Ca^{2+} channels because they can be activated by small depolarizations from relatively negative holding potentials. The current kinetics are fast and these channels exhibit rapid (and purely voltage-dependent) inactivation. In contrast to L-type channels, T-type channels are more Ni^{2+}-sensitive and less Cd^{2+}-sensitive. The T-type channels are also largely, if not completely, resistant to DHP antagonists. The single channel conductance is 15-17 pS. T-type channels are found in numerous excitable and non-excitable cells (Hess, 1990). The functions of T-type channels are to support pacemaker activity or Ca^{2+} entry at negative membrane potentials (Tsien *et al.*, 1988; Bean, 1989; Hess, 1990).

(iii) Other Types of Ca^{2+} Channels

N-type channels appear to be almost completely restricted to neurons (Tsien *et al.*, 1988). These channels are high-voltage activated but resistant to DHPs. They are largely, if not completely, blocked by ω-conotoxin (Tsien & Tsien, 1990). The P-type channel has only been found in cerebellar Purkinje cells and in squid giant synapses (Tsien & Tsien, 1990). P-type channels are also high threshold channels and insensitive to both DHPs and ω-conotoxin, but are sensitive to certain spider toxins (Bindokas & Adams, 1989; Llinás *et al.*, 1989; Cherksey *et al.*, 1990; Salzberg *et al.*, 1990). These two types of Ca^{2+} channels are not discussed in depth since this study does not deal with them.

(B) *Modulation of VDCC*

Voltage- and time-dependent Ca^{2+} channels can be modulated by neurotransmitters, hormones and drugs. Most of these agents modulate VDCC through alteration of channel gating and/or phosphorylation. This modulation may influence the electrical activity of cells and other cellular activities. Neurotransmitters and hormones usually modulate VDCC through G proteins. The G proteins can directly or indirectly (through intracellular second messengers) interact with VDCC (Yatani *et al.*, 1987a; Imoto *et al.*, 1988; Rosenthal *et al.*, 1988b). Therefore, a G protein-gated Ca^{2+} channel has been postulated by Brown and Birnbaumer (1988). This category of channel requires membrane depolarization and receptor activation (Brown & Birnbaumer, 1990). These channels are usually sensitive to agonists which activate membrane associated G proteins and act directly or indirectly. G proteins couple membrane receptors to ionic channels by a cytoplasmically independent, membrane-delimited pathway (three element system) or through cytoplasmic enzyme pathways (multiple element system) (Brown & Birnbaumer, 1990). In both cases, G proteins are required. The response in the three element system is faster than the multiple element system (Yatani & Brown, 1989). The three element system (direct pathway) does not require intracellular kinases. For example, purified G_s or α_s directly activates Ca^{2+} channels in vesicles from skeletal muscle T-tubules or cardiac sarcolemma which were incorporated into planar phospholipid bilayers (Yatani *et al.*, 1987a, 1988a). The multiple element system (indirect pathway) requires the generation of an intracellular cascade to respond to the binding of an agonist to its receptor, such as β-agonists binding to the β-adrenergic receptors (β-agonist---β-receptor---G_s---AC---cAMP---PKA---Ca^{2+}channel). The modulation of the voltage-dependent Ca^{2+} channels by G proteins is the main focus of this thesis.

1.2.2. ***Ligand-Gated Ca^{2+} Channels***

In addition to the voltage-dependent ion channels, another major group of Ca^{2+} channels is the agonist-gated (receptor-operated) ion channels. (Hille, 1989). Two subtypes make up this category: ligand-gated Ca^{2+} channels with indirect voltage-gating and ligand-gated Ca^{2+} channels without voltage-gating.

(A) ***Ligand-Gated Ca^{2+} Channels With Indirect Voltage-Gating***

The activation of this type of ligand-gated channels requires both receptor activation and depolarization. For example, the N-methyl-D-aspartate receptor (NMDA) channel opens in response to glutamate and allows significant Ca^{2+} entry as well as the movement of monovalent cations in both directions (inward and outward). The Ca^{2+} entry is strongly increased by depolarization that drives Mg^{2+} out of the channel and thereby relieves the block (Ascher & Nowak, 1987).

(B) *Ligand-Gated Ca^{2+} Channels Without Voltage Gating*

An example of this type of ligand-gated channel is the adenosine triphosphate (ATP) channel in arterial smooth muscle (Benham & Tsien, 1987b). It only activates in response to ATP. Unlike the voltage-dependent Ca^{2+} channels, the ligand-gated Ca^{2+} channels are not very selective for Ca^{2+}. However, these channels can be classified as ligand-gated Ca^{2+} channels since they allow a functional entry of Ca^{2+}.

1.2.3. *Modulation of VDCC by G Proteins in Four Types of Cells*

Although a large body of evidence demonstrated that G proteins modulate Ca^{2+} channel activity in a number of cells (Williamson, 1986; Rosenthal & Schultz, 1987; Spiegel, 1987), the mechanisms are still not well understood. Ca^{2+} channels vary from tissue to tissue and many experiments have shown that different cells have different G proteins and distinct Ca^{2+} channel regulatory mechanisms.

(A) *Neurons*

G proteins are responsible for the modulation of VDCC in neurons. In cultured dorsal root ganglion (DRG) neurons, 500 μM GTPγS potentiated the inhibitory effect of baclofen (a GABA receptor agonist) on the inward Ca^{2+} current (Scott & Dolphin, 1986). Similarly, noradrenaline and GABA-aminobutyric acid appeared to inhibit the inward Ca^{2+} current through a G protein and this effect was mimicked by GTPγS and blocked by pre-treatment with pertussis toxin (Hescheler *et al.*, 1988c). In pertussis toxin pre-treated neuroblastoma glioma hybrid cells (NxG cells), the inhibitory action of a synthetic opioid, which specifically activated receptors of the opiate δ-type, was restored by intracellular infusion of purified G proteins (G_i or G_o) (Hescheler *et al.*, 1987a). Serotonin, which activates a receptor on the snail neurone membrane, caused an increase in the intracellular concentration of cGMP which activated cGMP-Pk (protein kinase). This, in turn, caused phosphorylation, either of the Ca^{2+} channel or of G proteins, which may decrease Ca^{2+} channel currents. Hence, the inhibitory effect on Ca^{2+} channels which was mediated by G proteins has been demonstrated. In addition, Dolphin and Scott (1987) reported that intracellular application of GTPγS inhibited the voltage-dependent L-type Ca^{2+} channel current in DRG neurons and that this effect of GTPγS was inhibited by PTX. It has also been shown by Dolphin and Scott (1988) that photorelease of intracellular GTPγS from a photolabile "caged" precursor had concentration-dependent effects on the T-type Ca^{2+} channel current. At a concentration of 6 μM, GTPγS enhanced the current, but subsequent addition of GTPγS at a higher concentration (20 μM) inhibited the current. Only the inhibitory response was sensitive to pertussis toxin. Therefore, direct activation of G protein using GTPγS in the absence of agonists has been shown to affect VDCC.

(B) *Endocrine Cells*

In endocrine cells, G proteins may mediate modulation of VDCC. The inhibitory effects of calcium and Ca^{2+} channel agonists on PTH release were blocked in parathyroid cells treated with pertussis toxin (Fitzpatrick *et al.*, 1986). In a pituitary cell line, ATt-20 D16/16, somatostatin produced inhibition of the inward Ca^{2+} current which was not secondary to changes in cAMP levels (Kochi *et al.*, 1985). However, this somatostatin-mediated inhibition of the inward Ca^{2+} current became irreversible in the presence of 100 μM GTPγS. PTX treatment abolished this somatostatin effect on the inward Ca^{2+} current (Lewis *et al.*, 1986). Lewis *et al.* (1986) also reported that GTPγS alone produced a slow inhibition of the inward Ca^{2+} current that was only slightly affected by subsequent application of somatostatin. Thus, it was suggested that a G protein may mediate this inhibitory effect on Ca^{2+} channels in endocrine cells. However, Rosenthal *et al.* (1988a) have demonstrated that somatostatin inhibited the Ca^{2+} current, whereas LHRH stimulated the Ca^{2+} current in GH_3 cells. Both hormones act through distinct PTX-sensitive G proteins. Therefore, it is apparent that the effects of G proteins on Ca^{2+} channels are complicated.

(C) *Vascular Smooth Muscle Cells*

The β effect of noradrenaline on VDCC in vascular smooth muscle cells is inconsistant. The response varied from no effect to a 50% reduction (Benham & Tsien, 1988). The observed response was shown to be mediated by G protein and to be dependent on cAMP (Droogmans *et al.*, 1987). However, Zeng *et al.* (1989a,b) reported that the direct activation of G proteins by fluoride (F^-) and GTPγS, known stimulators of G proteins, increased the contraction of rat tail artery helical strips and that this increase was extracellular Ca^{2+} dependent. The contraction was induced by KCl or other agents, such as vasopressin or norepinephrine. This effect was partially blocked by the Ca^{2+} antagonist, nifedipine. It was also reported that the contraction was dependent on the concentration of applied GTPγS and was not affected by pretreatment with cholera toxin. Thus, it was proposed that G proteins (but not stimulatory G proteins) may be involved in the gating of voltage-dependent Ca^{2+} channels in vascular smooth muscle.

(D) *Cardiac Myocytes*

A large amount of work has been done to demonstrate that G proteins modulate VDCC in cardiac myocytes. During the last ten years, the concept of the direct or indirect modulation of Ca^{2+} channel currents by G proteins in cardiac cells became established through analysis of the biochemical steps involved (Birnbaumer *et al.*, 1985). Progress has been made possible by the availability of isolated cell preparations (Powell *et al.*, 1980; Isenberg & Klöckner, 1982) and new biophysical techniques, i.e. the patch clamp technique (Hamill *et al.*, 1981). Wang *et al.* (1991a) have reported that parathyroid hormone [bPTH-(1-34)] increased the L channel current in neonatal ventricular myocytes. The β-adrenergic stimulation of the L-type Ca^{2+} channel is the best-documented example of a hormonal effect on Ca^{2+} channels (Reuter, 1974; Tsien *et al.*, 1986). In guinea-pig and frog ventricular myocytes, β-agonists increased the inward Ca^{2+} current significantly via the G protein (G_s) (Trautwein & Hescheler, 1990).

This effect was then mediated by cAMP-dependent phosphorylation which served as an intracellular signal cascade (Nargeot *et al.*, 1983; Shuba *et al.*, 1990). Experiments on guinea-pig cardiac myocytes and bovine cardiac sarcolemmal vesicles incorporated into planar lipid bilayers showed a possible direct effect of $G_{s\alpha}$ on calcium channels that was independent of cAMP, ATP, cAMP activated protein kinase (PKA) or protein kinase C (PKC) (Yatani *et al.*, 1987a). GTPγS (100 μM), G_s (20-100 pM) or α_s increased the channel opening probability and prolonged the channel mean open time. Although G_s seems the most likely candidate to be a physiological regulator in cardiocytes, other G proteins cannot be excluded. Since the evidence for a G protein effect on VDCC in cardiocytes is sufficiently well documented, myocytes were used in the experiments to establish a standard for G protein involvement in the modulation of VDCC. GTPγS, a G protein activator was used in initial experiments. In addition, the maximal increase in Ca^{2+} current by G proteins seems to be species-dependent (Trautwein & Hescheler, 1990). The effect of GTPγS on the Ca^{2+} channel current in neonatal rat myocytes has not been previously reported. It is, therefore, interesting to compare the difference between neonatal rat and other species.

2. G Protein Coupling of Receptor (α_1-Adrenoceptor) to Effector (Ca^{2+} Channel)

A large number of cellular signal transduction processes arise from the combinations of many different types of agonists, receptors and coupling molecules such as the heterotrimeric G protein and protein kinases that connect receptors and effectors. The signal transduction mechanism may involve a single element that is both a receptor and an ion channel, or it may involve multiple elements including receptors, ion channels, G proteins and kinases (Brown, 1991). The number of known pharmacologically and/or molecularly distinct receptors coupled to effectors by G proteins has dramatically increased to 91 (Birnbaumer, 1990). Recent studies have uncovered new levels of complexity in the organization of G protein-mediated signal transduction. There are several possible mechanisms. A single G protein (G) could be activated by more than one receptor (R) and regulate more than one effector system (E) (R_1-G-R_2, E_1-G-E_2). A single receptor may activate more than one G protein and a single effector system could be regulated by more than one molecular species of G protein (G_1-R-G_2, G_1-E-$G2_2$). For example, in DRG neurons, neuropeptide Y (NPY) and bradykinin (BK) both inhibited Ca^{2+} current through a PTX-sensitive G protein. Purified α_0 could reconstitute the NPY effect whereas reconstitution of BK response required the combination of purified α_0 and α_{i2} (Ewald *et al.*, 1989). Hence, more targeted studies are required which deal with specific signal transduction pathways such as the example mentioned above to clarify the complex area of signal transduction.

2.1. α_1-Adrenoceptors in the Heart

2.1.1. *Occurrence and Distribution*

α-Adrenoceptors have been identified in the heart by physiological, pharmacological and direct radio-ligand binding experiments (Clark & Rattigan, 1986). Binding studies have shown that the specific subtype of α-adrenergic receptor in rat heart membranes is α_1 (Williams *et al.*, 1981). Studies using isolated myocytes also suggest that the α_1-adrenoceptor is the major α-adrenergic receptor in the heart (Buxton *et al.*, 1986).

2.1.2. *Functions*

The effects of catecholamines on the heart are mainly due to the stimulation of β-adrenoceptors. In the mid-1960s the first evidence was presented that α-adrenoceptors also mediate positive inotropism in the myocardium (Williamson *et al.*, 1964). In addition to inotropism, α-adrenoceptors also mediate chronotropism and dromotropism in the heart (Benft, 1990). Unlike the β-adrenoceptor-mediated response, the α_1-adrenoceptor-mediated positive inotropic effect is not accompanied by a shortening in time to peak force and relaxation time. The duration of the contraction is, instead, prolonged (Brücker *et al.*, 1985). The positive inotropic response to α-agonists is biphasic; there is a transient response followed by a lag and then a sustained response (Osnes *et al.*, 1978; Skomedal *et al.*, 1982; Otani *et al.*, 1988). The transient response occurs within 30 seconds while the sustained response takes several minutes to develop (Otani *et al.*, 1988). Although positive inotropic effects of α-adrenergic agonists have been reported in many species, some investigators have suggested that these effects are due to non-specific stimulation of β-adrenoceptors by the α-agonists used (Endoh *et al.*, 1978). For example, phenylephrine, a commonly used α agonist, is not absolutely specific for α-adrenoceptors (Schümann *et al.*, 1987). The reasons for the differences in response to α_1-adrenergic stimulation by phenylephrine may be attributed to variation among species. In rats, α_1-adrenergic receptor stimulation resulted in a greater response to phenylephrine when compared with dogs, calves and baboons. This was considered to be associated with the greater α_1-adrenergic receptor density in the rat heart (Shen *et al.*, 1989). Hence, the α-adrenergic responses in some species may be small or absent and the only responses produced by phenylephrine (at high concentrations) could be mediated by β-adrenoceptors because of the low α-adrenoceptor density. Although the precise role of α_1-adrenoceptors in the heart is still uncertain, they may assume greater physiological significance under conditions of β-adrenergic blockade, hypothyroidism and myocardial ischemia. In addition, the roles they play under physiological or pathological situations may vary with animal species.

2.1.3. *Ionic Mechanisms*

The ionic mechanism underlying the α_1-adrenoceptor-induced positive inotropic response is still controversial. A large body of evidence has revealed that the α_1-adrenergic response can be attributed to the suppression of an outward transient K^+ current (I_t) in rabbit atrial and ventricular cells (Fedida *et al.*, 1990), adult rat ventricular cells (Apkon & Nerbonne, 1988; Ravens *et al.*, 1989; Tohse *et al.*, 1990; Ertl *et al.*, 1991), adult rat atrial cells (Ertl *et al.*, 1991). Several investigators have presented evidence that an α_1-adrenoceptor activation-induced positive inotropic effect is not due to Ca^{2+} channel activation (Apkon & Nerbonne, 1988; Ravens *et al.*, 1989; Ertl *et al.*, 1991; Terzic *et al.*, 1992). It has also been shown that in feline ventricular myocytes, phenylephrine increased the Ca^{2+} channel current; but this effect was mediated by a β-adrenergic receptor since it was blocked by propranolol (Hartmann *et al.*, 1988). An explanation for this positive inotropic effect is that in physiological situations, the large transient outward current repolarizes the cell and accelerates I_{Ca} deactivation during the phase of rapid repolarization. When I_t is partially blocked by an α-agonist, I_{Ca} is larger and flows for a longer period of time as the onset of repolarization is delayed. Thus, it was proposed that the prolongation of the action potential which increases the influx of Ca^{2+} might be responsible for the positive inotropic response (Fedida *et al.*, 1990). However, it was also reported earlier that when β-adrenoceptors were blocked by propranolol, the positive chronotropic response to phenylephrine was enhanced by increasing the extracellular calcium concentration or by the use of the calcium channel activator Bay K 8644 (0.1 μM). The response was decreased by lowering the calcium concentration or by the use of the calcium antagonists verapamil, nifedipine and diltiazem (Rand *et al.*, 1986). The explanation of this data by Rand *et al.* (1986) was that the positive inotropic response to α_1-adrenoceptor activation is due to an increased influx of calcium through calcium channels. However, the results obtained by Rand *et al.* also could be explained by Fedida and co-workers' proposal that an increased influx of Ca^{2+} results from suppression of I_t (Fedida *et al.*, 1990). Nevertheless, it is also possible that phenylephrine has a direct effect on Ca^{2+} channels in some tissues. For example, in bovine heart, phenylephrine has been reported to increase the slow inward current without changing the outward K^+ current (Brückner & Scholz, 1984).

2.1.4. *Other Factors That Affect α_1-Adrenoceptor Responses*

Many factors have been shown to influence the positive inotropic response mediated via α-adrenoceptors. An essential factor is α_1-adrenoceptor density. It has been suggested that whether phenylephrine exerts its inotropic effects through α- or β-adrenoceptors depends upon the relative densities of α and β receptors (Chess-Williams *et al.*, 1990). Using a [^{3}H]-dihydroalprenolol binding assay, the density of α-adrenoceptors in rat ventricular cells was found to be four times greater than that of β-adrenoceptors (Chess-Williams *et al.*, 1990). The α_1-adrenergic receptor density in rat heart was the greatest when compared with that of baboon, calf or dog. The density of the receptors is usually associated with major physiological differences between these species in response to phenylephrine (Shen *et al.*, 1989). In addition, the rate at which isolated tissues are stimulated (Endoh & Schümann, 1975; Shibata *et al.*, 1980) and the bathing temperature (Kunos & Nickerson, 1977) have been shown to generate different responses to the activation of α-adrenoceptors. Therefore, it is necessary to consider various factors and to determine appropriate experimental conditions for demonstrating the optimal α_1-adrenoceptor inotropic response.

2.2. **G Proteins in the Rat Heart**

The ability of a cell to respond to a hormone or neurotransmitter depends not only on the number of receptors and effectors, but also on the amount and identity of G proteins present in the plasma membrane. Regulation of G protein levels is a potential point of control which would determine the ability of a cell to respond to a particular hormone. Changes in G protein levels have been demonstrated to occur during cellular differentiation (Watkins *et al.*, 1987) or organ development (Leutje *et al.*, 1988) parallel with changes in hormone responsiveness (Sibert *et al.*, 1990). It was observed that as early as fetal day 16, three substrates (45-, 47- and 52-KDa proteins) were identified which could be CTX-catalyzed ADP-ribosylated using [^{32}P]ADP-ribosylation of the α subunit of G protein as an assay (Kojma *et al.*, 1988). The use of immunoblotting analysis has confirmed that many types of G proteins are present in cardiac tissues. For example, neonatal rat cardiac myocytes contain $G_{s\alpha 52}$, $G_{i\alpha 1,3}$ and $G_{i\alpha 2}$ (Foster *et al.*, 1990). A PTX-insensitive G protein mediates α-adrenoceptor-induced positive chronotropic responses in neonatal rat heart, whereas in adult rat, a PTX-insensitive G protein mediates α_1-adrenoceptor-induced negative chronotropic responses, and a PTX-sensitive G protein mediates α_1-adrenoceptor-induced positive chronotropic responses (Han *et al.*, 1989; Rosen & Robinson, 1990). However, it is generally believed that different G proteins are responsible for distinct intracellular reactions. The differences in the tissue-specific expression of G protein subtypes suggest that the levels of individual G proteins may be important determinants of hormonal response in a given cell type (Sibert *et al.*, 1990). Since the G proteins coupled to α_1-adrenoceptors vary from the neonate to adult rat heart, the different positive inotropic and chronotropic effects induced by α-adrenergic agents in neonatal and adult rat can be partially explained by G protein variation.

2.3. Second Messenger Systems

2.3.1. *cAMP-PKA Pathway*

α-Adrenoceptors in cardiac tissue are exclusively of the α_1 subtype in that they are selectively blocked by prazosin and phentolamine and they are activated by phenylephrine and methoxamine (Hartzell, 1989). Radioligand binding studies with [^{3}H]-yohimbine did not provide any evidence for an α_2 subtype in rat heart (Buxton & Brunton, 1986). The α-adrenergic receptor density in rat heart is higher than β-adrenergic receptor density (Chess-Williams *et al.*, 1990). Even though stimulation of α_1- and β-adrenergic receptors both produced positive inotropic responses, stimulation of α-adrenoceptors did not elevate cAMP levels and usually decreased them (Benfy, 1973; Watanabe *et al.*, 1977; Buxton & Brunton, 1986). The α-adrenergic mediated decrease in cAMP levels was associated with a decrease in cAMP-dependent protein kinase (PKA) activity (Buxton & Brunton, 1986). This decrease in cAMP levels was also apparently related to activation of a cyclic nucleotide phosphodiesterase. The species of phosphodiesterase involved has not been identified. Therefore, it was proposed that ventricular myocytes have α_1-adrenergic receptors that bind agonists and that occupation of these receptors is functionally coupled by G proteins to decreased cellular cAMP via activation of cAMP phosphodiesterase activity.

2.3.2. ***PIP$_2$-PKC Pathway***

In many tissues α_1-adrenergic receptors are coupled to phosphoinositide metabolism (Exton, 1983; Nishizuka, 1984; Berridge, 1987) and this is also true in the heart (Brown & Johns, 1986). In many cell types, stimulation of α_1-adrenoceptors results in activation of phospholipase C which hydrolyses phosphatidyl inositol 4,5-bisphosphate

(PIP_2) to form inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DG). IP_3 has been shown to stimulate mobilization of internal Ca^{2+} stores by opening Ca^{2+} channels in the sarcoplasmic reticulum (Suarez-Isla *et al.*, 1988) and DG has been shown to stimulate protein kinase C (Nishizuka, 1984).

It has been shown that α_1-adrenoceptor stimulation of rat left ventricular papillary muscles by phenylephrine in the presence of propranolol resulted in rapid breakdown of PIP_2 and a triphasic inotropic response which occurred in a concentration-dependent manner (Otani *et al.*, 1988). The release of IP_3 was maximal within 30 seconds and remained high for at least 30 minutes. The IP_3 formation was associated with a rapid, but small increase in contractile force followed by a transient decline in the contractile force prior to the development of a sustained and more pronounced positive inotropic response. Inhibition of PI-4,5-P_2 hydrolysis by the α_1-adrenergic antagonist prazosin or the PI-4,5-P_2 phosphodiesterase inhibitor neomycin blocked all components of the inotropic response. Addition of 2,3-diphosphoglyceric acid (a competitive inhibitor of IP_3 phosphatase), combined with phenylephrine doubled the IP_3 formation and potentiated the initial phase of the inotropic response but had no effect on the sustained positive inotropic response. Nifedipine and Mn^{2+} did not block the transient inotropic response but inhibited the sustained positive inotropic response. α_1-Adrenoceptor stimulation resulted in restoration of the sustained responses in the high K^+-depolarized muscles during a time course similar to that of the development of the sustained positive inotropic response. Addition of phorbol-12,13-dibutyrate alone or in combination with caffeine or A23187 failed to produce a sustained positive inotropic effect, but pretreatment with this phorbol ester (1-100 μM) for 30 minutes resulted in a dose-dependent potentiation of an α_1-adrenoceptor-mediated sustained positive inotropic effect associated with an enhanced sustained response. Therefore, it appears that the positive inotropic effects mediated by cardiac α_1-adrenoceptor stimulation occur through the phosphodiesteratic cleavage of PIP_2 such that IP_3 produces transient inotropic effects by mobilizing intracellular Ca^{2+}. Diacylglycerol, along with cofactors that are also generated by α_1-adrenoceptor stimulation, evokes the sustained positive inotropic effect by potentiating slow Ca^{2+} channels through activation of protein kinase C (Otani *et al.*, 1988). The α_1-adrenoceptor stimulated IP_3 formation was also confirmed by Steinberg *et al.* (1989) in neonatal rat heart using a sensitive HPLC technique. However, it was reported that α_1-adrenergic modulation of I_t (transient outward K^+ current) did not occur via the IP_3/IP_4-dependent increase in intracellular Ca^{2+} (Braun *et al.*, 1990).

The correlation between the positive inotropic response to α_1-adrenoceptor and protein kinase C activation, as mentioned above, suggests that the PKC pathway may be involved. However, the role of PKC in response to α-adrenergic receptor stimulation of the heart is not well understood. In intact heart, it was found that phorbol esters produced a negative inotropic response in cardiac tissues which was opposite to the positive inotropic response to α_1-receptor stimulation (Leatherman *et al.*, 1987; Ruskoaho *et al.*, 1985; Dosemeci *et al.*, 1988). In contrast, a recent study showed that phenylephrine caused a concentration and time-dependent positive inotropic response in rabbit heart through PKC. This PKC activation was associated with increased phosphorylation of a 15 KDa sarcolemmal protein and a 28 KDa cytosolic protein in the myocardium (Talosi & Kranias, 1992). Braun *et al.* (1990) reported that their observations did not support a role for PKC in the α_1-modulation of I_t in adult rabbit atrial myocytes. In neonatal rat ventricular cells, phorbol esters were reported to increase Ca^{2+} current (Dosemeci *et al.*, 1988; Henrich & Simpson, 1988; Lacerda *et al.*, 1988). It remains to be determined whether these results reveal important information about the species of protein kinase C that is activated by different substances such as phorbol esters, or whether they merely represent side effects of phorbol esters (Hartzell, 1989). Other studies have demonstrated that purified skeletal muscle Ca^{2+} channels can be phosphorylated by purified protein kinase C, which results in the activation of this dihydropyridine-sensitive Ca^{2+} channel (Chang *et al.*, 1991; Gutierrez *et al.*, 1991).

2.4. The Pathological Significance of α_1-Adrenoceptors

Under normal physiological conditions cardiac α_1-adrenoceptors may not be important. However, altered α-adrenergic receptors may be of critical importance in the pathogenesis of arrhythmias and in influencing myocardial oxygen demand and myocardial cell viability after ischemic insult (Kagiya *et al.*, 1991). It was found that α-adrenoceptors are also related to myocardial hypertrophy (Takeda *et al.*, 1991). During ischemia, it has been observed that α-adrenoceptor responsiveness was enhanced (Moore & Parratt, 1973) and the number of α-adrenergic receptors was increased as well (Corr *et al.*, 1991). In agreement with this result, Kayiga *et al.* (1991) have demonstrated that prolonged hypoxia increased α_1-adrenoceptor density without changing antagonist affinity in neonatal rat ventricular cells. Studies in the cat have shown an increase in α_1-adrenoceptor density after 30 minutes of acute ischemia (Corr *et al.*, 1981) and this may be associated with the pathogenesis of ventricular arrhythmias (Sheridan, 1986). This was supported by the report that α-adrenoceptor antagonists reduced the incidence of ventricular fibrillation during ischemia and reperfusion of the heart, while α-adrenoceptor agonists increased the incidence of arrhythmias during ischemia and reperfusion in the dog, cat and rat (Flores & Sheridan, 1991). In association with the α-adrenoceptor density increase in ischemia, it was observed that β_1-adrenoceptor density declined in the heart (Kagiya *et al.*, 1991). All of the above evidence is suggestive of the involvement of α_1-adrenoceptors in the pathology of the heart.

Although these findings suggest that hypoxia results in a marked increase in surface receptors on isolated myocytes, the questions remain of whether the exposed receptors are coupled to intracellular events and what their overall relation to arrhythmogenesis is in the ischemic heart. Recent studies have revealed that the α_1-adrenergic receptor in myocytes is in fact coupled intracellularly to an increase in IP_3 and that hypoxia results in a marked reduction in the concentration of norepinephrine required to elicit an increase in IP_3 (Heathers *et al.*, 1989). The increase in α_1-adrenergic responsiveness in the ischemic heart is not simply a function of the increase in α_1-adrenergic receptors, but there also appears to be an enhancement of coupling to intracellular mechanisms, possibly mediated by increased coupling to G proteins and activation of phospholipase C.

Compared to adult rat ventricular myocytes, neonatal myocytes possess some characteristics similar to ischemic myocytes, for example, the high density of α_1-adrenoceptors. Because of the difficulty in preparation of ischemic cells for electrophysiologic studies, this study of α_1-adrenergic responsiveness in neonatal rat myocytes may provide some clues in the search for the mechanisms of ischemic pathogenesis.

3. Comparison of the Properties of Adult and Neonatal Ventricular Myocardium

3.1. Response to α_1-Adrenoceptors

It has been reported that α_1-adrenoceptor stimulation by concentrations of 10^{-10} to 10^{-8} M phenylephrine had a negative chronotropic effect on isolated ventricles from adult rats but had a positive chronotropic effect on neonatal ventricles (Drugge *et al.*, 1985). These results are related to the maturation of autonomic innervation to the neonatal rat myocytes (Rosen & Robinson, 1990). Non-innervated cultures of neonatal myocytes exhibited a positive chronotropic response to α-adrenoceptor stimulation. Addition of sympathetic neurons to neonatal myocytes in co-culture induced the development of a negative chronotropic response to α-adrenoceptor stimulation which was similar to that found in intact ventricles from adult rats. Steinberg *et al.* (1985) confirmed that the chronotropic responsiveness to α_1-adrenergic agents changed from positive in the neonate to negative in the adult rat. In addition, it was found that primary cultures of neonatal rat ventricular myocytes expressed only a positive α_1-adrenergic chronotropic response (Steinberg *et al.*, 1985). The response to phenylephrine can be inhibited by the α_1-adrenergic inhibitor, prazosin. It has been demonstrated that the positive inotropic action of phenylephrine was increased in myocardium isolated from young adult rats as compared to neonatal animals (Nakanish *et al.*, 1989). Other than positive inotropism, α_1-adrenergic stimulation in neonatal rat ventricular cells also has two other major trophic effects. The first effect is the stimulation of an muscle cell increase in size (hypertrophy). The second effect is induction of spontaneous contraction. The hypertrophic response is mediated by α_1-adrenoceptors. The beating response requires both α_1- and β_1-adrenergic receptor stimulation. α_1-Stimulation alone produced enlarged cells that did not contract spontaneously. α_1 Plus β_1 adrenergic stimulation induced contractile activity even when protein synthesis and hypertrophy were inhibited (Simpson *et al.*, 1986).

3.2. Density of α_1-Adrenoceptors

It has been suggested that the positive inotropic action of α_1-adrenoceptors was decreased in myocardium from neonate to adult. In agreement with this, the α_1-adrenoceptor density was reported to decrease during development in rabbit, rat and dog heart but showed no change in affinity (Partilla *et al.*, 1982; Buchthal *et al.*, 1987; Nakanishi *et al.*, 1989; Rosen & Robinson, 1990). It was shown that neonatal rat ventricular myocytes contained predominantly α_1-adrenoceptors rather than β-adrenoceptors (Karliner *et al.*, 1985). Therefore, it is possible that the major positive inotropic response in the neonatal rat heart may be due to the activation of α_1-adrenoceptors.

3.3. Action Potentials and Ion Channels

3.3.1. *Action Potential*

The transmembrane action potential of single myocytes is characterized by rapid depolarization, a plateau and a slow repolarization. The initial depolarization is due to an increase in Na^+ permeability (activation of fast Na^+ channels). This is followed by a slow increase in Ca^{2+} permeability (activation of slow Ca^{2+} channels) which produces the plateau. Repolarization after the plateau is due to an increase in K^+ permeability. Two components, I_t (transient outward current) and I_{k1} (inward rectifier K^+ current), make up this K^+ current which contributes to repolarization. I_t is responsible for the rapid initial phase of repolarization and I_{k1} is responsible for changes in the final phase of repolarization and in genesis of the resting potential (Kilborn & Fedida, 1990).

In rat ventricular muscle, the action potential undergoes a major developmental change after birth (Kilbone & Fedida, 1990). Ventricular myocytes from prenatal and newborn rats have an action potential with an elevated plateau similar to that of other mammalian species (Couch *et al.*, 1969; Bernard, 1975). The adult rat ventricular action potential, however, is unique when compared to the action potential in ventricular cells of other mammals. The action potential in rat ventricular cells has a duration of less than 100 ms (Apkon & Nerbonne, 1988). An I_t antagonist, 4-AP, prolongs all action potentials but has almost no effect on 1-day myocytes. The effect of 4-AP is most prominent in adult myocytes. The postnatal shortening of the long neonatal rat action potential occurs over a time period of three weeks (Langer *et al.*, 1975). During the process of ontogeny the channels are expressed sequentially. In embryonic rat heart cells, the first channels to appear are the channels responsible for the slow inward current which carry Ca^{2+}; their role in contraction *per se* is evident. The second channels to be expressed are the Na^+ channels which improve propagation in cardiac muscle. The final channels to be expressed are the K^+ channels which shorten the action potential duration and permit a greater variation [illegible] cardiac rhythm (Bernard, 1975).

3.3.2. *Na^+ Channels*

It has been demonstrated in embryonic chick heart that the Na^+-specific tetrodotoxin (TTX)-sensitive, fast conductance mechanism is absent or non-functional for 2 to 3 days after incubation. The appearance of the fast Na^+ channels occurred on day 5 and the density increased during the remainder of development (Sperelakis *et al.*, 1975). These processes are associated with protein synthesis (Dehaan *et al.*, 1975).

3.3.3. *K^+ Channels*

Kilborn and Fedida (1990) reported that two major K^+ channels changed during the course of development in rat cardiac myocytes: I_{k1}, the inwardly rectifying background potassium current and I_t, the transient outward current. It was shown that I_{k1} decreased in magnitude by a factor of approximately three during the first ten postnatal days. The reduction in I_{k1} during maturation is the cause of the slowing of repolarization during the final phase of the action potential in adult rat heart. This change in I_{k1} with maturation was partially mediated by changes in the kinetic properties of I_{k1}. The voltage-dependence of inactivation of I_{k1} was shifted towards more positive potentials during development. In contrast, in rabbit ventricular myocytes, I_{k1} exhibited a pattern of increasing density with age (Huynh *et al.*, 1992). Consistent with the results of Huynh *et al.* (1992), it has been shown that in embryonic chick myocytes, both channel density and the single-channel conductance of I_{k1} increased with age (Josephson & Sperelakis, 1990). In another case, Chen *et al.* (1991) have demonstrated that I_{k1} channel density and opening probability are approximately the same in neonatal and adult cardiac myocytes. Therefore, it appears that the expression of I_{k1} during development depends on the species. Additional studies are needed in this area.

In contrast to I_{k1}, I_t is often absent, or apparent only at positive potentials in newborn rat myocytes (Kilborn & Fedida, 1990). There is approximately a four-fold increase in I_t current density from day 1 to day 10 neonatal rat ventricular myocytes. An antagonist of I_t, 4-AP, prolonged the action potential duration at various ages. 4-AP has a small effect on newborn rat ventricular action potentials. This suggests that the voltage-activated transient outward current (I_t) is of little functional importance in these cells.

3.3.4. *Ca^{2+} Channels*

The densities and properties of Ca^{2+} channels in neonate and adult rat ventricular myocytes appear to be different. The amplitude of the Ca^{2+} channel current (I_{Ca}) in neonatal cells was larger than that seen in the adult (Cohen & Lederer, 1988). A change in the voltage-dependence of inactivation of the Ca^{2+} channel current occurs during development. This results in a calcium window current which is present at birth and then disappears during subsequent development. This window current may be the mechanism responsible in part for the long plateau phase of the ventricular action potential in newborn rat myocytes. Two agents, ryanodine and caffeine, were used to interfere with sarcoplasmic reticulum (SR) function of ventricular cells in order to investigate the difference in I_{Ca} seen in neonatal and adult rat myocytes. Neither ryanodine nor caffeine alters I_{Ca} in neonatal myocytes. In contrast, both increase the transient and the steady-state components of I_{Ca} in adult ventricular myocytes (Cohen & Lederer, 1988). A possible explanation for the differences in I_{Ca} arises from the difference in anatomy of the two cell types. Calcium channel proteins in the plasma membrane can interact with functioning SR, probably through a spanning protein (Cohen & Lederer, 1988). Using electron microscopy, it has been observed that neonatal cells lack a fully functioning SR while adult cells have sharp sarcolemmal borders, fully developed sarcomeres with T-tubules and an SR membrane where Ca^{2+}-release channels are located. The spanning protein serves as a mechanical link between plasma membrane and SR. Since ryanodine has two binding sites on the SR membrane (Inui *et al.*, Fleischer, 1987), it can alter I_{Ca} through the spanning protein in the case of adult rat myocytes. In neonatal myocytes, due to the lack of SR, the effect of ryanodine was absent (Cohen & Lederer, 1988). Hence, it is likely that calcium channels are more important at this stage of development. On the other hand, in rabbit ventricular myocytes, it was found that I_{Ca} density increased from neonatal to adult and the intrinsic properties of the Ca^{2+} channel did not appear to change during development (Huynh *et al.*, 1992). The density of nitrendipine-sensitive Ca^{2+} channels increased rapidly during fetal development, reaching a plateau at 7-10 days after birth (Huynh *et al.*, 1992). The diversity in the pattern of developmental changes in Ca^{2+} channels is probably a result of differences between species.

3.4. **Structure**

The cardiac ultrastructure of rats and mice shows a striking decrease in the amount of glycogen and a marked increase in the number of myofibrils during development. The myocardium is characterized by definitive T-tubules and nexi 10 days after birth (Hirakow & Gotoh, 1975). In neonatal rat myocytes there is a well-defined nucleus, but the cytoplasm is not highly organized and sarcomeres are not readily visible although there are partially organized myofilaments and glycogen with groups of mitochondria. T-tubules are scarce or absent and there is no recognizable SR in these cells. However, in adult rat myocytes the cytoplasm is well organized with bands of myofilaments interspersed with mitochondria. The sarcomere structure is easily discernible. There are invaginations of the sarcolemma along the Z-lines which represent the sarcolemmal outlet of the T-tubule system. Well developed SR can also be seen (Cohen & Lederer, 1988). There are striking structural differences between neonatal and adult cells, only some of which were listed here. This anatomical diversity may also contribute to the functional differences discussed in this thesis.

3.5. **G Proteins**

It is obvious from the above discussion (section 3.1) that α_1-adrenergic responsiveness is different in the neonatal and adult rat. The levels of various G proteins and the roles that G proteins have in adrenergic action on the heart are also different in neonatal and adult rats. For example, G_s isolated from neonatal ventricles contained almost exclusively $G_{s\alpha 52}$ whereas adult ventricles contained predominantly $G_{s\alpha 45}$. Likewise, neonatal rat heart cells were found to contain significant amounts of both $\alpha_{i\alpha 2}$ and $\alpha_{i\alpha 1,3}$

while adult ventricles contained smaller amounts of these proteins (Foster *et al.*, 1990). In agreement with this, Kojima *et al.* (1988) showed that the level of one PTX substrate increased abruptly in 4 day old rats and then decreased to the final adult level. In the neonate, PTX did not have any effect on the α_1-adrenergic-mediated positive chronotropic response, whereas in the adult, the α_1-adrenergic-mediated negative chronotropic response was completely converted to a positive response after PTX treatment. Hence, a PTX-insensitive G protein is present in the neonatal and adult rat heart, and a PTX-sensitive G protein is linked to the negative chronotropic response in adult rat heart (Han *et al.*, 1989). In support of this, it was also shown that a PTX-insensitive G protein was involved in the positive inotropic or chronotropic effect mediated by α_1-adrenoceptors (Schmitz *et al.*, 1987; Braun *et al.*, 1990). Therefore, it is likely that the positive inotropic response to α_1-adrenergic agonists is mediated by a PTX-insensitive G protein in either neonatal or adult rat hearts. Parallel increases in sympathetic innervation and the presence of the 41 KDa PTX substrate were observed in fetal through neonatal dog hearts (Danilo, 1985; Rosen *et al.*, 1988). This increased 41 KDa PTX substrate coincides with the change from α-adrenergic excitation to inhibition of automaticity (Rosen & Robinson, 1990).

4. Objectives

4.1. To Measure the Direct Effect of GTPγS on Ca^{2+} Channels in Four Cell Types

The four cell types used in this thesis were GH_3 cells (endocrine cells), N1E-115 cells (peripheral neurons), smooth muscle cells from rat tail artery (vascular smooth muscle cells) and neonatal ventricular cells (cardiac myocytes). Under the same experimental conditions, the effect of GTPγS on different Ca^{2+} channels in these four cell types were compared. Two toxins, PTX and CTX were used to verify the modulatory effect of G proteins on VDCC and further identify the G proteins. The reasons for using this approach are as follows: (1) In different tissues, the effect of GTPγS on different Ca^{2+} channel current varies. GTPγS may increase, decrease or cause no change in Ca^{2+} channel currents. Therefore, the interaction of G proteins and Ca^{2+} channels was investigated in four types of cells under similar experimental conditions. (2) Most studies dealing with G proteins have focused on the hormonal control of Ca^{2+} channels. In order to determine the direct activation of G proteins bypassing receptor activation, GTPγS was used intracellularly to activate G proteins in the four cell types. (3) With the help of two bacterial toxins, PTX and CTX, not only the involvement of G protein modulation of VDCC can be confirmed, but also the classification and/or identification of G protein will be determined. The results from these studies support the hypothesis that G proteins modulate VDCC in various tissues. In addition, these results provide the basis for the additional studies described in the subsequent chapters of this thesis.

4.2. To Characterize the Specific G Protein and the Second Messenger Pathway Which Couples the α_1-Adrenoceptor to the L-Type Ca^{2+} Channel in Neonatal Rat Ventricular Myocytes

The initial part of these studies characterizes the ionic basis of the α_1-adrenergic effect on neonatal rat myocytes. The α_1-adrenergic agonist phenylephrine was used to establish that α_1-adrenergic stimulation induced an increase in the L-type Ca^{2+} current in neonatal rat ventricular myocytes. The effect of phenylephrine on the L-type Ca^{2+} current was carefully studied since it is contradictory to some of the recent work in adult animals. Subsequent studies describe the complete mechanism starting from the characterization of the G protein to the description of the second messenger cascade which results in the phosphorylation of the L-type Ca^{2+} channel. The G protein activator, GTPγS, and the G protein inhibitor, GDPβS, were used along with phenylephrine to establish that a G protein mediated the effect. The use of two toxins, PTX and CTX, provided evidence which helped to determine the identity of the G protein. The involvement of PKA and PKC pathways in response to α_1-adrenergic stimulation which caused the increase in L-type Ca^{2+} channel current was studied using agonists and antagonists of PKA and PKC.

4.3. To Compare the Effect of α_1-Adrenoceptor Activation on L-Type Ca^{2+} Channel Currents in Neonatal and Adult Rat Myocytes

The effect of the α_1-adrenergic agonist phenylephrine on the L-type Ca^{2+} channel current was compared in neonatal and adult rat ventricular myocytes under the same experimental conditions in order to determine the differences between these two types of myocytes in response to α_1-adrenoceptor stimulation.

CHAPTER II. MATERIALS AND METHODS

1. Cell Preparations

1.1. GH_3 Cells

GH_3 cells (derived from rat pituitary adenoma) were obtained from the American Type Culture Collection (Rockville, MD, USA). A stock line was maintained by growing a monolayer of cells in 250 ml plastic culture flasks in 12 ml of Ham's F-10 medium with 10% horse serum and 2.5% fetal bovine serum (FBS) in a humidified atmosphere consisting of 95% room air and 5% CO_2 at 37°C. The cells were dissociated from the flasks by incubating with 0.04% trypsin-EDTA for 5-10 minutes at room temperature and were then washed and centrifuged twice to remove the trypsin. The cells were split at a ratio between 1:4 to 1:10, replated and used for electrophysiological studies 6-8 hours after replating. The cells were spherical in shape after replating and the diameter ranged between 10-15 μm.

1.2. N1E-115 Cells

Neuroblastoma cells (N1E-115, undifferentiated murine neuroblastomas) were a gift from Dr. D. Schiff, Department of Pediatrics, University of Alberta. A stock of cells was maintained in a flask in 90% DMEM (Dulbecco's modified Eagle medium, Gibco, Grand Island, NY, USA) with 10% FBS and antibiotics PNS (penicillin, neomycin and streptomycin). The culture plates were maintained in a temperature controlled (37°C), humidified atmosphere consisting of 95% room air and 5% CO_2. The stock cells were gently triturated with a pasteur pipette before direct transferr to 3 ml petri dishes and the experiments were performed 20 hours after plating. The cells were spherical in shape with a diameter of 30-50 μm.

1.3. **Vascular Smooth Muscle Cells**

Vascular smooth muscle cells were isolated from the rat tail artery as reported previously (Wang *et al.*, 1989). The tail artery from male Sprague-Dawley rats (180-250 g) was excised and the connective tissue removed. The artery was cut into small pieces and then incubated with Hank's balanced salt solution (HBSS) (Gibco) at 4°C for 30 minutes. The tissue was treated with enzyme solution I containing collagenase/dispase 1.5 mg/ml (Boehringer Mannheim, GmbH, Laval, Quebec), elastase 0.5 mg/ml (type II-a, Sigma Chemical Co., St. Louis, MO, USA), trypsin inhibitor 1 mg/ml (type I-s, Sigma) and bovine serum albumin 2 mg/ml (Sigma) for 1 hour at 37°C. The tissue was further digested with enzyme solution II containing collagenase 1 mg/ml (Sigma, type II), trypsin inhibitor 0.3 mg/ml and bovine serum albumin 2 mg/ml for 1 hour at 37°C. The tissue was then mechanically agitated and the harvested cells were plated onto petri dishes. The calcium concentration of the incubating solution was increased to 2 mM by the stepwise addition of Ca^{2+}. The cells were stored for 4 hours at 4°C in DMEM without fetal calf serum (FCS) but containing insulin (0.8 U/ml, Sigma), penicillin (100 U/ml, Sigma) and streptomycin (0.1 mg/ml, Sigma). The solution was then replaced by DMEM containing

10% FCS to facilitate the attachment of the cells. The recordings were made 8-36 hours after replating. The cells were spherical in shape and the diameter was about 15 μm.

1.4. Ventricular Cells

1.4.1. *Neonatal Rat Ventricular Cells*

Single ventricular cells were isolated from neonatal rat hearts by enzymatic dispersion and mechanical disruption as previously reported (Wang *et al.*, 1991a; Liu *et al.*, 1992b). The hearts were removed rapidly under sterile conditions from the chest cavity of neonatal rats (3-4 days old). To exclude the atrium, only two-thirds of the lower part of the ventricles were used. The ventricles were cut into small pieces. These pieces were incubated with HBSS containing 1 mg/ml trypsin (Sigma) and 1 mg/ml bovine serum albumin (Sigma) at 37°C for 20 minutes. The incubating solution was discarded to remove all possible connective tissue. The tissue pieces were then placed in the same enzyme solution in a shaker (RotoMix, type 48200, Thermolyne, Dubuque, IA, USA) for 15 minutes at 37°C. There were six successive incubations under the same conditions. The tissue pieces were triturated with a pasteur pipette after each incubation. The enzyme solution after the remaining tissue pieces were removed was collected and mixed with DMEM at a ratio of 5:7. The solution was then centrifuged and the cells were resuspended in DMEM containing 10% FBS and 0.01% PNS. After filtration of resuspended solution through 200 μm nylon mesh to remove large pieces of undigested tissue, the dispersed cells were incubated at 37°C in an atmosphere of 5% CO_2-95% room air for 2 hours to facilitate the attachment of the fibroblasts to the culture dishes.

After the fibroblasts were attached, the ventricular myocytes were removed and dispersed into 35 mm petri dishes and kept in an incubator in a humidified atmosphere containing of 95% room air and 5% CO_2 at 37°C. The experiments were performed 16-36 hours after the cells were plated. During this period, the cells were spherical in shape and contracted in response to various stimuli. The cells started to beat rhythmically about 48 hours after incubation. In a standard physiological solution, action potentials could be generated in these cells. The diameter of the cells varied from 20 to 30 μm.

1.4.2. *Adult Rat Ventricular Cells*

Single ventricular cells were prepared from male Sprague-Dawley rats (180-200 g) according to the method of Ravens *et al.* (1989). The rat was anaesthetized by intraperitoneal injection of sodium pentobarbital (80 mg/Kg). The heart was removed rapidly. The aorta was cannulated and the heart was perfused via the coronary arteries for 5 minutes with a Ca^{2+}-free solution consisting of (in mM) NaCl 100, KCl 10, $MgSO_4$ 5.0, KH_2PO_4 1.2, glucose 20, taurine 50, MOPS [3-(N-morpholino)propanesulfonic acid] 10 and 1 mg/ml bovine serum albumin (Sigma), which was adjusted to a pH of 7.2 and aerated with 95% O_2-5% CO_2 at 37°C. This was followed by 10-15 minutes of perfusion with a collagenase solution composed of 1.0 mg/ml collagenase (type IV, Sigma) in a Ca^{2+}-free solution at a rate of 10 ml/min at 37°C. Following collagenase digestion, the aorta and atria were trimmed away and the left ventricle was cut open and the ventricular tissues were incubated in fresh collagenase solution at 37°C, and gently agitated. The myocytes were harvested by decanting the solution from the remaining ventricular tissue.

The myocytes were then washed and centrifuged at 20 g for 1 minute at room temperature. This procedure was repeated four times in Ca^{2+}-free solution. The solution was filtered through a 200 μm nylon mesh and diluted to about 3×10^5 cells/ml. The Ca^{2+} concentration in the myocyte suspension solution was gradually increased to 2 mM by the addition of Ca^{2+} in four steps. The cells were rod shaped and displayed cross striations after the isolation procedure. Cells were approximately 20-30 μm in width and 80-100 μm in length. These cells contracted in response to various stimuli.

2. Ventricular Tension Studies

Ventricles excised from the neonatal rat (3-4 days old) were suspended in Sawger-Bartlestone chambers and electrically stimulated using depolarizing pulses (square wave, 2 ms duration). The voltage was set 20% above threshold and a Grass stimulator SD 9 (Grass Instruments Co., Quincy, MA, USA) was used to deliver the depolarizing pulses at a frequency of 4 Hz. The developed tension was recorded using a Gould force transducer and a Gould recorder (Gould Inc., Cleveland, OH, USA). The ventricles were kept under isometric conditions in an oxygenated Krebs solution at 37°C. The composition of the Krebs solution was as follows (in mM): NaCl 121.9, KCl 4.7, KH_2SO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 21.0 and glucose 11. The contractile responses to the agonists were studied in the normal Krebs solution. Cumulative dose-response curves for phenylephrine were determined by adding 0.01 ml of the drug solution. The concentration in the organ bath was increased in steps of 1 log unit. When a steady level of the developed tension was reached, the next higher concentration was applied. The equilibration time for each concentration was usually 15-20 minutes.

3. Patch Clamp

3.1. Voltage Clamp

The inward current through voltage-activated calcium channels was recorded using the whole-cell version of the patch clamp technique (Hamill *et al.*, 1981).

3.1.1. *Electrodes*

The patch electrodes were pulled by a two stage electrode puller (Narishige Scientific Instrument, Lab MF-83, Tokyo, Japan) and heat polished from a thin walled glass capillary (OD=1.2, ID=0.9, FHS, Brunswick, Maine, USA) with a resistance ranging between 2-6 MΩ when filled with internal solution.

3.1.2. *Solutions*

Inward Ca^{2+} currents were measured using 20 mM Ba^{2+} as the charge carrier. Tetrodotoxin (TTX) and zero Na^+ were used to eliminate inward Na^+ current. TTX was used at a concentration of 10^{-6} M in GH_3 cells, neuroblastoma cells and vascular smooth muscle cells, and 20 μM in ventricular cells. Because Na^+ channels in ventricular cells have a low affinity for TTX (Kd=1 μM) (Cohen *et al.*, 1981) and these cardiac cells have a large number of Na^+ channels (Bean, 1985), a higher concentration of TTX is required. Cs^+ was used internally and Ba^{2+} was used externally to block K^+ current. Ba^{2+} was the charge carrier and measured inward currents represent the inward movement of Ba^{2+} through Ca^{2+} channels. The bath solution contained (in mM): TrisCl 105, $MgCl_2$ 0.8, KCl 5.4, $BaCl_2$ 20, TTX 0.001-0.02 and HEPES 10. The bath solution for all GTPγS experiments contained (mM): tetraethylammonium chloride (TEA) 150, $MgCl_2$ 0.8, KCl 5.4, $BaCl_2$ 20, TTX 0.001 and HEPES 10. The internal solution contained (in mM): Cs_2-aspartate 70, HEPES 20, EGTA 11, $CaCl_2$ 1, $MgCl_2 \cdot 6H_2O$ 5, K-succinate 5 and glucose 5. ATP-Na_2 5, creatine phosphokinase 50 U/ml and phosphocreatine-Na_2 20 mM were added to the pipette solution to reduce current run-down (Forscher & Oxford, 1985). In current clamp experiments, the external solution contained (in mM): NaCl 140, KCl 5.4, $MgCl_2 \cdot 6H_2O$ 1.2, $CaCl_2 \cdot$ 1.8, HEPES 10 and glucose 10. The internal or pipette solution contained (in mM): KCl 130, EGTA 11, $CaCl_2$ 1, $MgCl_2 \cdot 6H_2O$ 2, NaCl 10, ATP-Na_2 4, HEPES 10 and glucose 5. All solutions were filtered (0.22 μm) before use. The osmolarity was adjusted to 310-330 mOsm using sucrose, and the pH was adjusted to 7.2-7.4 using $Ba(OH)_2$, NaOH or HCl.

3.1.3. *Experimental Procedures*

Spherical cells with a clear border were usually chosen for electrophysiological experiments. The junction potentials were balanced out by applying a d.c. offset voltage until the recorded current became zero. The electrode position was adjusted using a Narishige manipulator. Once a successful gigaohm seal was achieved, additional suction was applied until the membrane patch was broken and access gained to the interior of the cell. A successful rupture of the patch was accompanied by an increase in the current noise and a large capacitance-charging current transient in response to the 10 mV command. The currents were filtered using a low pass filter set at a frequency of 4 KHz (Axon Instruments, Foster City, CA, USA). All experiments were performed at room temperature (20 - 22°C).

3.1.4. *Compensation of Leakage Current and Series Resistance*

On-line leakage subtraction was implemented using the P/N protocol in pClamp software. Two hyperpolarizing subpulses were used and it was assumed that these subpulses did not elicit any active currents and thus any currents recorded were due to passive leakage. In early experiments in this thesis, leakage correction was applied manually. The input resistance was checked regularly. Inward Ba^{2+} current was recorded with measured seal resistances of approximately 10 GΩ or higher. When neuroblastoma cells and ventricular cells were used, the seal resistance was usually around 50 GΩ. Cells were never used if the seal resistance was lower than 10 GΩ. The series resistance in the majority of these experiments was less than 5 MΩ. The peak inward current in most of the cells was usually less than 500 pA and the voltage error was 2 mV or less so that series resistance was usually not a problem. When the voltage error was greater than 2 mV, series resistance was compensated using Axopatch electronics. It was usually impossible to compensate completely.

3.1.5. *Membrane Current Measurements*

The membrane currents were measured using two different amplifiers, a List EPC-7 patch clamp amplifier (List Electronics D-6180 Darmstadt, Fed. Rep. Germany) or an Axopatch-1B patch clamp amplifier (Axon Instruments). The data was sampled using pClamp software (version 5.5) and an Axolab 1100 analog-to-digital converter or a TL-1 DMA interface (Axon Instruments) and stored on a floppy disc using a Zenith computer. The current was monitored using a digital oscilloscope (Nicolet Instrument Co., Madison, Wisconsin, USA). When the currents were recorded on the floppy drive of a digital oscilloscope, data analysis and leakage correction were carried out manually. The majority of the experiments described in this thesis were analysed using pClamp software. The membrane current was always measured as the peak inward current (leakage corrected) unless otherwise stated.

3.1.6. *Capacitive Current and Space Clamp*

The capacitive current transients usually settled within 100 to 500 μs in most of the preparations. This was fast enough to separate either T- or L-type Ca^{2+} channel currents. Adequate space-clamp was judged under the experimental conditions by the following criteria. 1) The capacitive current decayed quickly (Byerly & Hagiwara, 1982). 2) When the membrane was depolarized to various levels, the amplitude of the peak current increased gradually with increasing depolarization in the negative slope region of the current-voltage (I-V) relationship without showing any detectable threshold phenomena (Ogata & Narahashi, 1989). 3) A partial block of the membrane current by conditioning depolarizing pulses did not change the time to peak of the inward current

(Ogata *et al.*, 1989). Cells were discarded if these parameters were not satisfied. Adult rat ventricular cells are large and rod shaped. These features could give rise to spatial and temporal clamp problems. However, when the membrane was sealed and ruptured, the cell contracted into a round shape and the duration of the capacitive current became small enough to be acceptable (less than 2 ms). Only experiments with adequate space clamp were used. In addition, the adult rat ventricular myocytes were used only to investigate the effect of phenylephrine on the magnitudes of L-type channel current but not changes in kinetics.

3.1.7. *Intracellular Ca^{2+} Concentration*

The estimated concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) was calculated using three conservation equations and eight equilibrium reactions (Stockbridge, 1987). 11 mM EGTA and 1 mM Ca^{2+} in the internal solution were used, which was calculated to correspond to a pCa=8 (Fenwick *et al.*, 1982). It has been reported that $[Ca^{2+}]_i$ was usually around 10^{-7} M when the cell was perfused with a solution buffered to any level of Ca^{2+} from $9x10^{-7}$ to below 10^{-8} M (Byerly & Moody, 1984). Therefore, in our experiments, the pCa was estimated to be between 7 and 8. With this concentration of intracellular Ca^{2+}, the response of the cell to agonists involving an intracellular second messenger cascade should be functional since Ca^{2+} dependent events would not be greatly affected. In addition, this concentration of $[Ca^{2+}]_i$ (less than 10^{-6} M) did not have an effect on the inactivation of the inward Ca^{2+} current (Byerly & Moody, 1984).

3.1.8. *Administration of Drugs and Washout of Drugs*

In most experiments, a 250 ms depolarization test pulse of increasing amplitude was applied at a frequency of 1 Hz. In adult rat ventricular cells, the depolarization test pulse was longer, 400 ms. Studies of the α-adrenoceptor effect usually started 5 minutes after rupture of the membrane, i.e, when the inward current reached the steady-state. Only those cells with a stable current (less than 5% decrease) 5-7 minutes after rupture of the membrane were chosen for further studies. The rate of run-down of the inward current in this case was usually negligible for 20 minutes onward (Wang *et al.*, 1989). When the inward current decayed too quickly after adminstration of the drug because of deterioration of the seal or run-down, the results were discarded. The current-voltage relationship was plotted and used as a control. The effects of the drugs were usually recorded every 5 minutes at a frequency of 3 Hz. Although some drugs shifted the peak current amplitude in the I-V relationship, the effect of the drug on the inward current was compared using the same test pulse amplitude. This technique was only used to generate the concentration dependence of the drugs. In some experiments, after the effect of the drug reached a steady-state, the bath solution was exchanged by wash-out.

3.2. **Current Clamp**

Current clamp experiments were carried out using an Axopatch 1B ($\beta=1$) patch clamp amplifier (Axon Instruments). The membrane potential was set to -80 mV. Action potentials were evoked at 10 second intervals by 15 ms superthreshold depolarizing constant current pulses delivered through the recording pipette. The action potentials before and after phenylephrine (at 5 minute intervals) were recorded on floppy discs associated with the Nicolet oscilloscope. The records were superimposed during the plotting procedure and the action potentials were compared before and after phenylephrine administration. The experiments were performed at room temperature.

4. Modulation of G Proteins

4.1. Intracellular Application of GTPγS and GDPβS

The non-hydrolysable GTP analogue GTPγS [guanosine-5'-O-(3-thiotriphosphate)], which was used as a G protein activator, and the non-hydrolysable GDP analogue, GDPβS [guanosine-5'-O-(2-thiodiphosphate)], a G protein inhibitor, were added to the pipette solution to modulate G proteins. In experiments in which the effect of GTPγS on Ca^{2+} channel currents in GH_3 cells, N1E-115 cells and vascular smooth muscle cells was measured, the current recordings were made every 30 seconds using the same magnitude test pulses. Time 0 was defined as the time at which the inward current (initial current, I_0) was established. This was taken as the time at which the K^+ current was blocked. This usually takes approximately 1 minute. The current (I) recordings at different times were compared with the initial current (I_0) and plotted against the time. Such a comparison was used because the absolute current amplitudes varied substantially from cell to cell. Since GTPγS is not membrane permeable and the G proteins are located on the inner side of the membrane, intracellular application of GTPγS is required. Therefore, two groups of cells, i.e, with the presence or absence of GTPγS in the internal solution, were used to determine the involvement of G protein in the modulation of Ca^{2+} channel currents. In other experiments, when the mechanism of phenylephrine was studied, the drug was added to the bath solution 5 minutes after rupture of the membrane with GDPβS or GTPγS present or absent from the pipette solution. The purpose of these experiments was to investigate the involvement of G proteins in the α_1-adrenoceptor agonist-enhancement of the L current. The experiments were carried out in this manner because changes in the current amplitude were observed to develop at the first few minutes after the replacement of the perfusate and a complete replacement of the intracellular ionic composition took place on the average in 5 minutes (Doroshenko *et al.*, 1982). Therefore, cells were allowed to equilibrate with the contents of the patch pipette for 5 minutes before recording was begun (Dolphin *et al.*, 1989).

4.2. **Flash Photolysis**

Caged-GTPγS {guanosine-5'-O-(3-thiotriphosphate), 3-s-[1-(2-nitrophenyl)ethyl] thio ester} (Molecular Probes Co., Eugene, OR, USA) was used to determine the effect of GTPγS on Ca^{2+} channel currents. The caged compound was diluted with the pipette solution on the day of use and kept at 0°C. After commencing the whole cell recording, cells were allowed to equilibrate for at least 5 minutes with the pipette solution containing the caged compound. Photolysis was then effected using a xenon flashlamp system (Hi-tech Scientific Limited, Wiltshire, England). The light guide was positioned 15 mm above the surface of the bath. The pulses used had an energy of 148 J and were approximately 1 ms in duration (Dolphin *et al.*, 1988). Usually, 2-3 pulses were sufficient to release adequate GTPγS.

4.3. Pretreatment of Cells with Toxins

In order to verify that the effect of GTPγS acted via G proteins and to further identify the G proteins responsible for the regulation of Ca^{2+} channels, pertussis toxin (PTX) and cholera toxin (CTX) were used (Ui, 1984; Gilman, 1987). Cells were incubated with 200 ng/ml PTX or 1 μg/ml CTX for 12-16 hours (Dophin & Scott, 1987). PTX and CTX were dissolved in 1-5% BSA (bovine serum albumin) solution as stock solution. They were diluted before addition to the media.

5. Application of Second Messengers

In order to determine the effect of α_1-adrenoceptors on the L-type Ca^{2+} channel current via the intracellular second messenger system, several activators and inhibitors were used. Adenosine-3',5'-cyclic monophosphothioate isomers Sp-cAMPs and Rp-cAMPs (Biolog, La Jolla, CA, USA) were used to exclude the possible involvement of the cAMP-PKA phosphorylation pathway. Sp-cAMPs (1 mM), a strong protein kinase A (PKA) activator, and Rp-cAMPs (100 μM), a PKA inhibitor (Eckstein *et al.*, 1976) were added to the pipette solution. 4β-phorbol 12-myristate, 13-acetate (PMA) (200 nM) and 1-oleoyl-2-acetyl-rac-glycero (OAG) (60 μM) (Sigma) were used to rule out the involvement of the protein kinase C (PKC) pathway. PMA and OAG are both activators of PKC. It has been reported that cells pre-treated with 500 nM PMA for 7-9 hours would down-regulate the level of endogenous PKC. It is believed that prolonged stimulation of PKC by phorbol esters leads to a loss of the enzyme through proteolytic degradation (Henrich & Simpson, 1988). Another alternative approach used was to block endogenous PKC activity. Staurosporine (100 nM) (Sigma) has been reported to be more potent than other inhibitors of PKC (Tamaoki *et al.*, 1986). Therefore, staurosporine was used in the experiments to study the effect of phenylephrine on the L-type Ca^{2+} channel current.

6. Drugs

GTPγS and GTPβS were purchased from Boehringer Mannheim GmbH. Phenylephrine, prazosin, propranolol, clonidine, yohimbine, staurosporine, nifedipine, NiCl, $LaCl_3$, OAG, PMA, pertussis toxin and cholera toxin were all purchased from Sigma. Rp-cAMPs and Sp-cAMPs were purchased from Biolog. Bay K 8644 was purchased from Calbiochem Co. (La Jolla, CA, USA). Prazosin was dissolved in distilled water by sonication. PMA, OAG, nifedipine and Bay K 8644 were dissolved in ethanol. The final concentrations of ethanol (0.03%) had no effect on the Ca^{2+} channel current. Other drugs were all dissolved in distilled water. The required concentrations of the drugs were obtained by adding 10 μl of drug stock solution to the 3 ml static bath. Only one experiment was conducted per dish to avoid any possible desensitization to the drugs. Approximately 2 minutes were required for the stock drug solution to distribute evenly within the dish and produce the responses. As required in some experiments, the agents were washed out by replacement of the bath solution.

7. Data Analysis

Data are given as mean±S.E.M., except for original traces and voltage-current relationships. The data obtained after drug administration are expressed as a percentage of the control values. The concentration-response curves were fitted to a Michaelis Menton equation (Hill equation) using a Marquardt-Levenberg least squares algorithm. The responses were normalized to give the maximal response which was considered to be 100%. The half-maximal response was calculated from the equation. The steady-state activation or inactivation of the Ca^{2+} channel current was fitted by the Boltzmann equation $V=\{V_{max}/[1+\exp(v_h-x)k]\}$. In this equation, V_h represents the holding potential, v is the potential at which one half of the calcium channels are inactivated or activated and k is the slope factor. The half activation and inactivation voltages were calculated using this equation. The Student's t test (paired or grouped) was used to estimate the statistically significant differences between the control cells and the cells treated with drugs. In the case of multiple comparisons, analysis of variance or Bonferroni analysis (Milliken & Johnson, 1984) was used.

CHAPTER III. THE EFFECT OF GTPγS ON CALCIUM CHANNEL CURRENTS IN FOUR TYPES OF CELLS

1. Introduction

In order to compare the effect of direct activation of G proteins on various Ca^{2+} channel currents in different tissues under similar experimental conditions, four types of cells were chosen. GTPγS, a G protein activator, was applied intracellularly to activate G proteins. In GH_3 cells, rapidly and slowly inactivating Ca^{2+} channel currents were separated and the effect of GTPγS on both channel components was investigated. Moreover, PTX and CTX were used to verify the effect of GTPγS on Ca^{2+} currents through activation of the G proteins and to further characterize the types of G proteins. For VSMC and cardiac myocytes, T- and L-type Ca^{2+} channel currents can be separated as shown elsewhere (Bean *et al*, 1985; Bean, 1986; Benham *et al.*, 1987a). However, a detailed separation of T- and L-type Ca^{2+} channel currents was not conducted due to the experimental conditions involved. The effects of GTPγS on total (T and L current) and L current were studied in these two types of cells. No further G protein characterization was performed in these cells. For neuroblastoma cells, both L- and T-type Ca^{2+} channels were identified (Wang *et al.*, 1990). However, under normal culture conditions, only T channels were observed. The expression of the L-type channel require special conditions, i.e., the cells have to be treated with 2% DMSO for one month. The study of the modulation of the L-type current in neuroblastoma cells was not included in this chapter because DMSO treatment may change some properties of the channel and G proteins. Since GTPγS activation of G proteins did not modulate the T channel current, toxins were not used in the experiment to characterize the G proteins.

2. Experimental Design

The whole cell version of the patch clamp technique was used in these experiments. GTPγS or caged-GTPγS, G protein activator, was added to the pipette solution. The following is a list of the experiments conducted on the four cell types.

2.1. **GH_3 Cells**

1) Characterization and separation of the rapidly and slowly inactivating currents.
2) Effect of GTPγS on Ca^{2+} channel currents.
3) Effect of GTPγS on Ca^{2+} channel currents in the presence of toxins (PTX and CTX).

2.2. **Neuroblastoma Cells**

1) Characterization of the T-type Ca^{2+} channel current.
2) Effect of GTPγS on the inward Ca^{2+} channel current.

2.3. **Vascular Smooth Muscle Cells**

1) Characterization of Ca^{2+} channel currents in VSMC.

2) The effect of GTPγS on the inward Ca^{2+} channel currents.

2.4. Neonatal Rat Ventricular Myocytes

1) Characterization of Ca^{2+} channel currents in ventricular cells (neonatal rat).

2) Effect of GTPγS on inward Ca^{2+} channel currents.

3) Effects of GTPγS on steady-state activation and inactivation of L-type Ca^{2+} channel current.

4) Effect of caged-GTPγS on Ca^{2+} channel current.

5) Comparison of total inward Ca^{2+} channel currents and L-type Ca^{2+} channel current.

3. Results

3.1. GH_3 Cells

3.1.1. *Characterization and Separation of the Rapidly and Slowly Inactivating Currents*

In a GH_3 cell, from a holding potential of -80 mV, a test pulse (250 ms duration) to -10 mV produced an inward Ca^{2+} current consisting of two components. One of the components inactivated rapidly and the other component inactivated slowly, as has been reported previously (Dubinsky & Oxford, 1984; Simasko *et al.*, 1988). Fig. III-1 shows the two components of the inward current measured in a GH_3 cell. On the left, the original current records show an inward current. The I-V relationship on the right was plotted from the current records shown on the left. The inward current was activated at -50 mV. There is a small current activated between -50 to -20 mV, indicating the presence of more than one type of Ca^{2+} channel current. The peak of the I-V relationship occurred at +20 mV, suggesting that the slowly inactivating current component was predominant in this cell. Fig. III-2 shows the typical slowly inactivating current component in a GH_3 cell. The original current records shown in Fig. III-2 were activated by depolarizing the cell to the test potentials indicated next to each current record from a holding potential of -40 mV. The original current records indicate that the current consisted of the slowly inactivating component. The current was activated at -20 mV and the peak occurred at +30 mV. The slowly inactivating component was inhibited by nifedipine and increased by Bay K 8644 (data not shown). Therefore, using different holding potentials, the two components of the inward Ca^{2+} channel current can be determined. The current records in Fig. III-3 show that the rapidly inactivating current component was completely inactivated at 100 ms after the beginning of the test pulse (holding potential of -80 mV), while the slowly inactivating component was essentially unchanged. When the holding potential was shifted to -40 mV and a test pulse to -10 mV was applied, the rapidly inactivating component was absent but the second peak current was almost the same as that obtained at 100 ms from a holding potential of -80 mV. The current observed at 100 ms from the beginning of the pulse was, therefore, used as a measure of the slowly inactivating current component (holding potential of -80 mV). The rapidly inactivating current component was obtained by subtracting the slowly inactivating current component from the first or early peak current. These calculations were used as an approximate means of separating the rapidly and slowly inactivating current components.

3.1.2. *Effects of GTPγS on Ca^{2+} Channel Currents in GH_3 Cells*

The intracellular application of GTPγS produced an increase in the Ca^{2+} channel current 2-3 minutes after the inward current reached a steady state. Fig. III-4 shows the inward currents evoked by test potentials to -10 mV from a holding potential of -80 mV in two cells. The test depolarizations were applied at the time indicated in the figure. The control cell was dialysed with normal pipette solution and the GTPγS cell was dialysed with a pipette solution containing GTPγS. In the cell dialysed with normal solution, the magnitude of the inward Ca^{2+} current changed very little showing some run-down at the end of 5 minutes. The inward current in the cell dialysed with GTPγS increased and then subsequently decreased with time. This was not due to run-down of the current, since the control cells did not show such significant run-down. In order to confirm this preliminary data, population studies were carried out. Experiments using groups of cells confirmed the effect of GTPγS on the Ca^{2+} channel current (Fig. III-5). The effect of GTPγS was not specific but significantly increased both the rapidly and slowly inactivating Ca^{2+} channel current components. The separation of the rapidly and slowly inactivating Ca^{2+} current components was performed using the approach described above (see Fig. III-3). The filled circles represent cells treated with GTPγS and the open circles represent control cells. The current ratios (currents at different times were normalized to the initial current, I/I_0) were calculated and plotted against time. The change was time-dependent. The Ca^{2+} channel currents increased after the inward current was established, and reached their peak at 3-4 minutes. This indicated that the GTPγS had diffused into the cell and activated G proteins. After 4-5 minutes, the inward current declined with time. It appears that under these experimental conditions, GTPγS first activated Ca^{2+} channel currents and subsequently decreased Ca^{2+} channel currents. The inhibitory effect of GTPγS has previously been reported in GH_3 cells (Lewis *et al.*, 1986).

3.1.3. *Effects of GTPγS on the Ca^{2+} Channel Current in the Presence of Toxins*

Two toxins, PTX and CTX, were used to ADP-ribosylate G proteins. In these experiments, all cells were pretreated for 12 hours with either PTX (200 ng/ml) or CTX (1 μg/ml). GTPγS (500 μM) was applied intracellularly to some cells and these were compared to the control cells (no GTPγS in the pipette).

In cells pretreated with CTX, the excitatory effect on the Ca^{2+} channel current induced by intracellular application of GTPγS was still observed (Fig. III-6). Fig. III-6 shows the current evoked from a holding potential of -80 mV to a test pulse of -10 mV in two cells. Both cells were pretreated with CTX. Fig. III-7 shows that combined data from two groups of cells supported the preliminary data. The slowly and rapidly inactivating Ca^{2+} channel current components showed the same pattern: GTPγS increased both Ca^{2+} channel current components in cells pretreated with CTX. However, after 5 minutes, the inward currents did not decrease with time as in cells not pretreated with CTX, indicating that the subsequent decrease in Ca^{2+} currents caused by GTPγS was not simply due to run-down of the currents. These results suggest that GTPγS still increased both the rapidly and slowly inactivating components in GH_3 cells in the presence of CTX, but that the decline of the Ca^{2+} channel current at 4-5 minutes induced by GTPγS was absent. This indicates that a CTX-sensitive G protein might be responsible for this decrease.

After pretreatment of cells with PTX, the effect of GTPγS on Ca^{2+} channel current was abolished (Fig. III-8). The original current records from two individual cells, one with GTPγS (500 μM) in the pipette and one without GTPγS in the pipette are shown in Fig. III-8. Both cells were pretreated with PTX. Fig. III-9 shows the effect of GTPγS on the two Ca^{2+} channel current components in a group of cells pretreated with PTX. This figure shows combined data from a group of cells. PTX abolished the effect of the GTPγS induced-increase in both the rapidly and slowly inactivating Ca^{2+} current components. These results show that GTPγS was ineffective in increasing the Ca^{2+} channel currents in the presence of PTX, indicating that a PTX-sensitive G protein may be responsible for this increase. Addition of the toxins PTX (200 ng/ml) or CTX (1μg/ml) to the bath solution during recording had no effect on the Ca^{2+} channel currents. Bonferroni analysis (Milliken & Johnson, 1984) showed there is no cross significance among the three control groups (i.e, the control group without toxin pretreatment and the two control groups with PTX and CTX pretreatment, respectively) (α=0.05/90).

Fig. III-1.

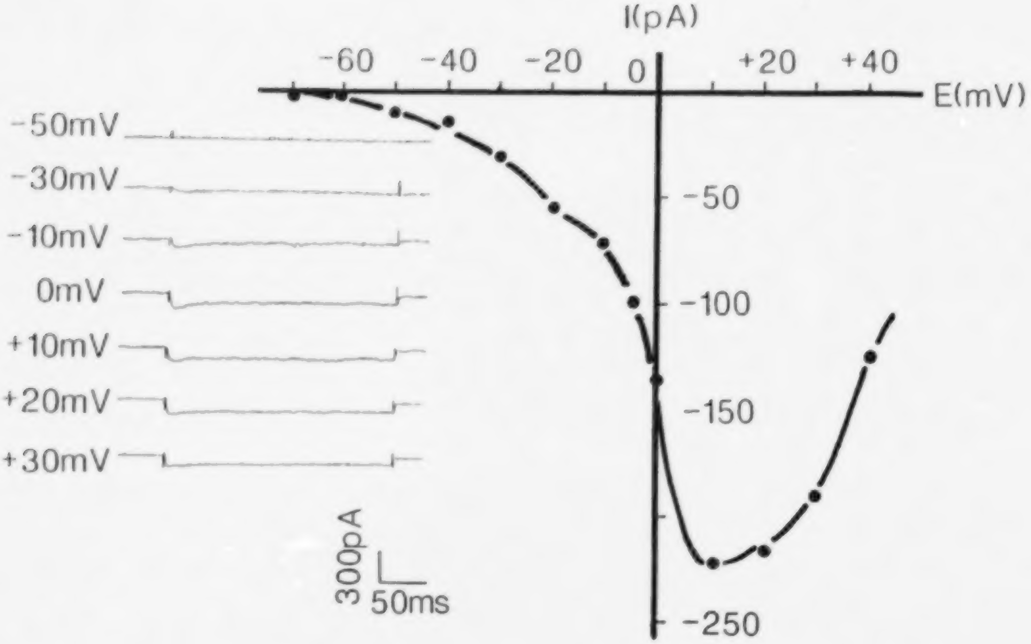


Fig. III-1. Original current records and the current-voltage relationship of the total inward current (calcium channel) in a GH_3 cell. Current records shown at the left were evoked by a 250 ms test pulse from a holding potential of -80 mV to the test potentials indicated next to each record (leakage was not corrected). The I-V relationship (on the right) shows an inward current which activated at -50 mV. The peak amplitude occurred at approximately +20 mV. The cell expressed both the rapidly and slowly inactivating current components.

Fig. III-2.

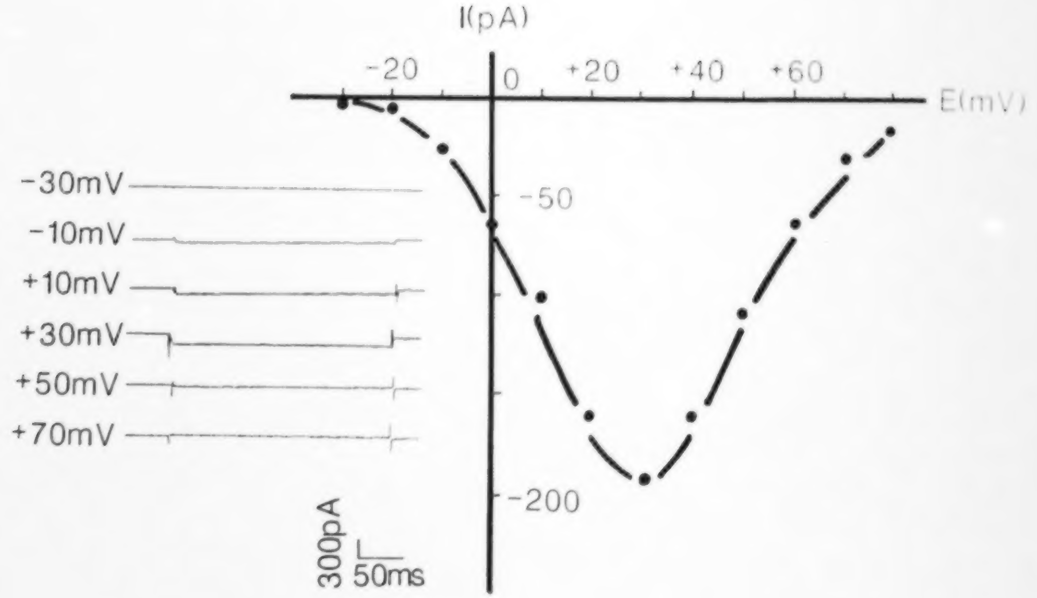


Fig. III-2. Original current records and current-voltage relationship of the slowly inactivating calcium channel current in a GH_3 cell. The current records shown on the left were evoked by a 250 ms test pulse from a holding potential of -40 mV to the test potential indicated next to each record (leakage was not corrected). The current records did not show appreciable inactivation. The inward current was activated at -30 mV and the peak amplitude occurred at +30 mV, suggesting that this is the slowly inactivating current.

Fig. III-3.

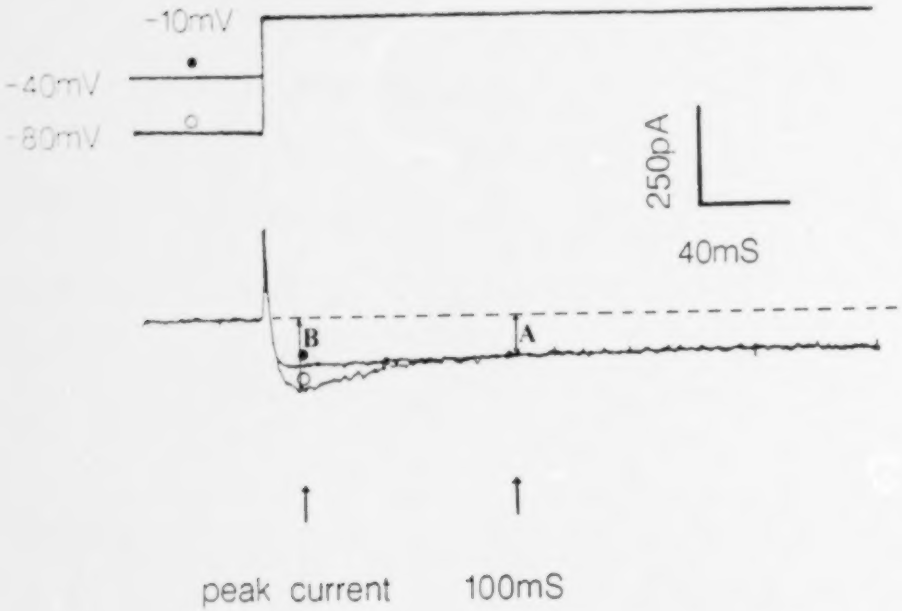


Fig. III-3. Separation of the rapidly and slowly inactivating Ca^{2+} channel current components in a GH_3 cell. When the holding potential was -80 mV depolarizing the cell to a test potential of -10 mV activated an inward current which consisted of two components. One component showed no significant inactivation after 100 ms. When the cell was depolarized to a test potential of -10 mV from a holding potential of -40 mV, only the slowly inactivating component of Ca^{2+} channel current was present. The magnitude after the two inward currents activated from the two holding potentials (-80 and -40 mV) at 100 ms from the beginning of the depolarization was the same. The current observed 100 ms after the beginning of the pulse (HP=-80 mV) was therefore used as a measure of the slowly inactivating component of the inward current. The rapidly inactivating current component was taken as the peak current (B) minus the current component at 100 ms (A). Therefore, the rapidly inactivating component=B-A. Leakage current was not subtracted in the original records and was very small for this set of experiments.

Fig. III-4.

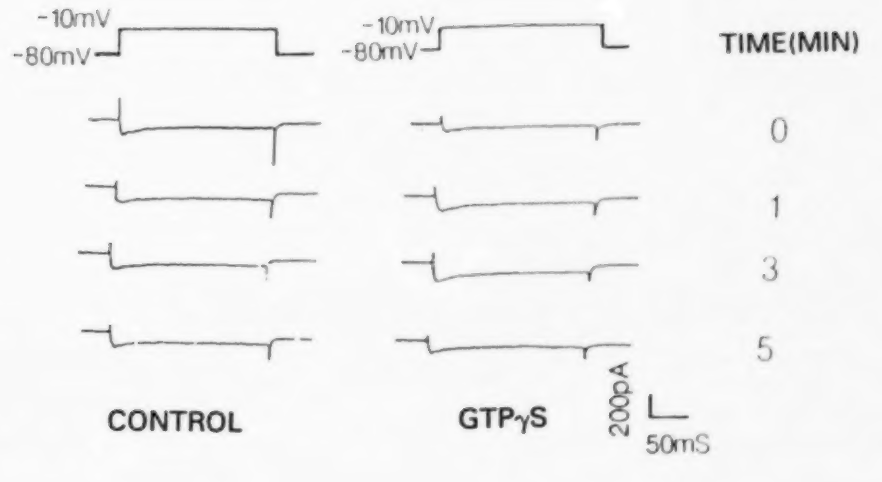
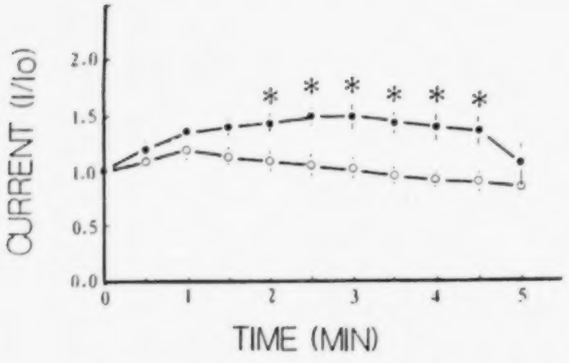


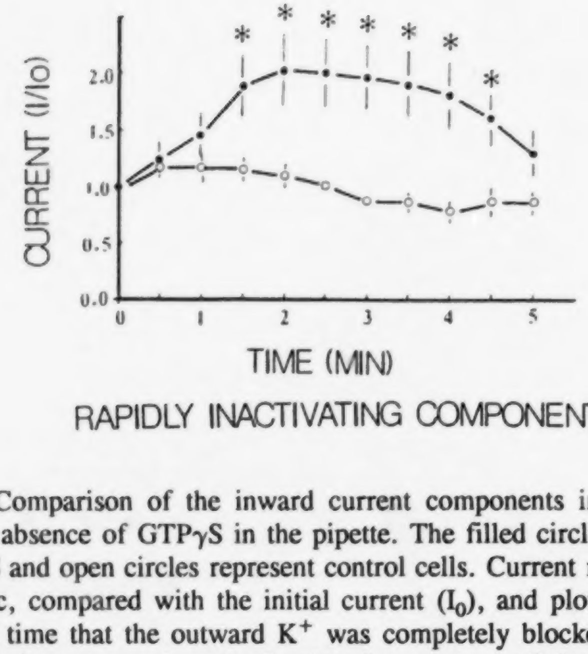
Fig. III-4. GTPγS modulated Ca^{2+} channel currents. Original current records obtained from two cells (control and GTPγS-treated) at 0, 1, 3 and 5 min after the control current was established. The current was activated by depolarizing the cell to -10 mV from a holding potential of -80 mV. Leakage current was not subtracted.

Fig. III-5.

CONTROL



SLOWLY INACTIVATING COMPONENT



RAPIDLY INACTIVATING COMPONENT

Fig. III-5. Comparison of the inward current components in groups of cells in the presence or absence of GTPγS in the pipette. The filled circles represent cells treated with GTPγS and open circles represent control cells. Current recordings (I) were made every 30 sec, compared with the initial current (I_0), and plotted against time. I_0 was taken at the time that the outward K^+ was completely blocked by Cs^+ (see Methods section 4.1), i.e., approximately 1 min after rupture of the cell membrane. GTPγS significantly increased both rapidly and slowly inactivating current components with time. The data is plotted as mean±S.E.M. (n=10). At each time interval the control and GTPγS data were tested for statistical significance using Bonferroni analysis. The Ca^{2+} channel current reached its peak at 2-3 min and the declined after 4-5 min. $*P<0.05$.

Fig. III-6.

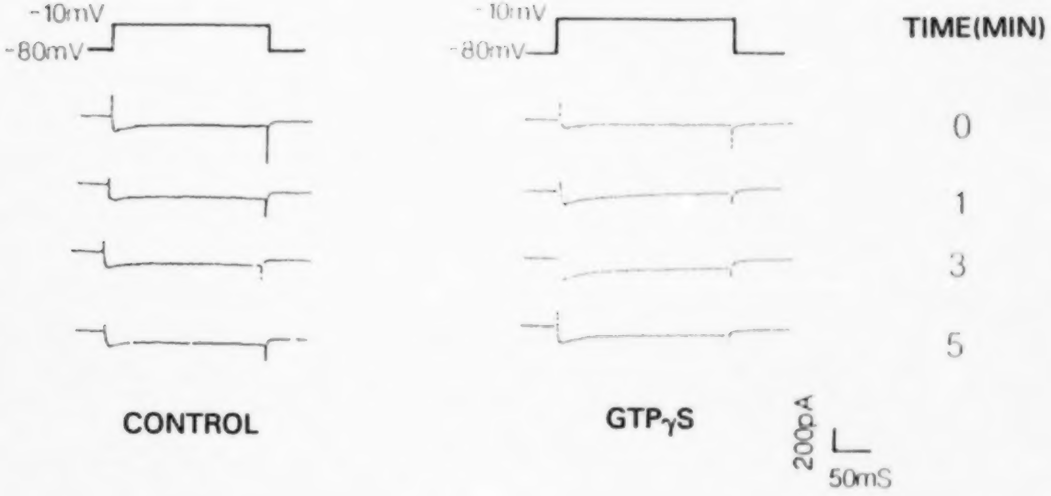
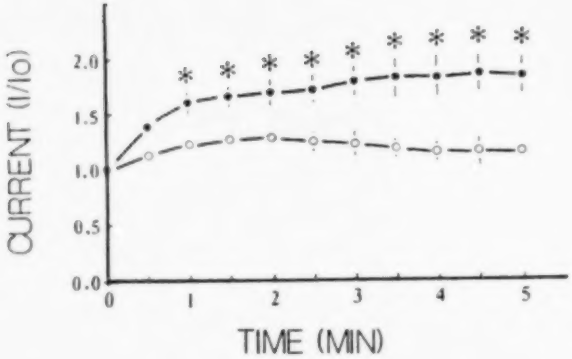


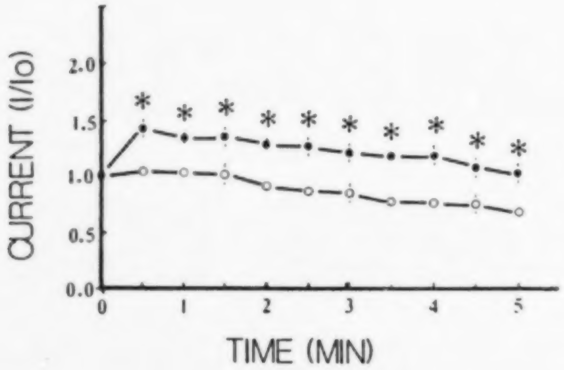
Fig. III-6. Original current records from two cells, one with GTPγS in the pipette and one without GTPγS in the pipette. These two cells were both pretreated with cholera toxin. The inward currents were activated from a holding potential of -80 mV to a test potential of -10 mV. Cholera toxin abolished the effect (increase) of GTPγS on inward Ca^{2+} currents (leakage was not corrected).

Fig. III-7.

CHOLERA TOXIN



SLOWLY INACTIVATING COMPONENT



RAPIDLY INACTIVATING COMPONENT

Fig. III-7. Comparison of groups of cells pretreated with cholera toxin. The data are expressed in the same manner as described in Fig. III-5. Cholera toxin abolished the GTPγS induced decrease (at 4-5 min) in Ca^{2+} channel currents (both rapidly and slowly inactivating Ca^{2+} channel current components) (n=9). However, the GTPγS induced increase in Ca^{2+} channel currents was unchanged.

Fig. III-8.

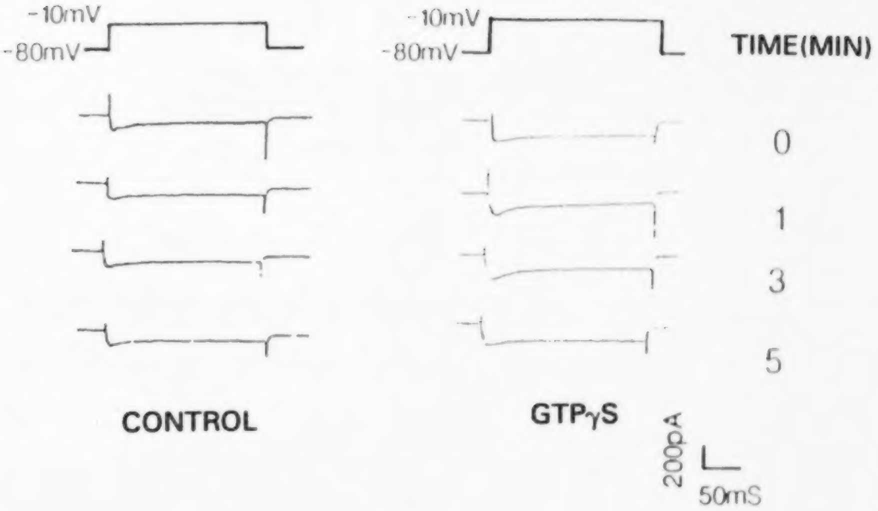


Fig. III-8. Original current records from two cells, one with GTPγS in the pipette and one without GTPγS in the pipette. These two cells were both pretreated with pertussis toxin. The inward currents were activated from a holding potential of -80 mV to a test potential of -10 mV. Pertussis toxin abolished the GTPγS-induced increase in Ca^{2+} channel currents. (leakage was not corrected).

Fig. III-9.

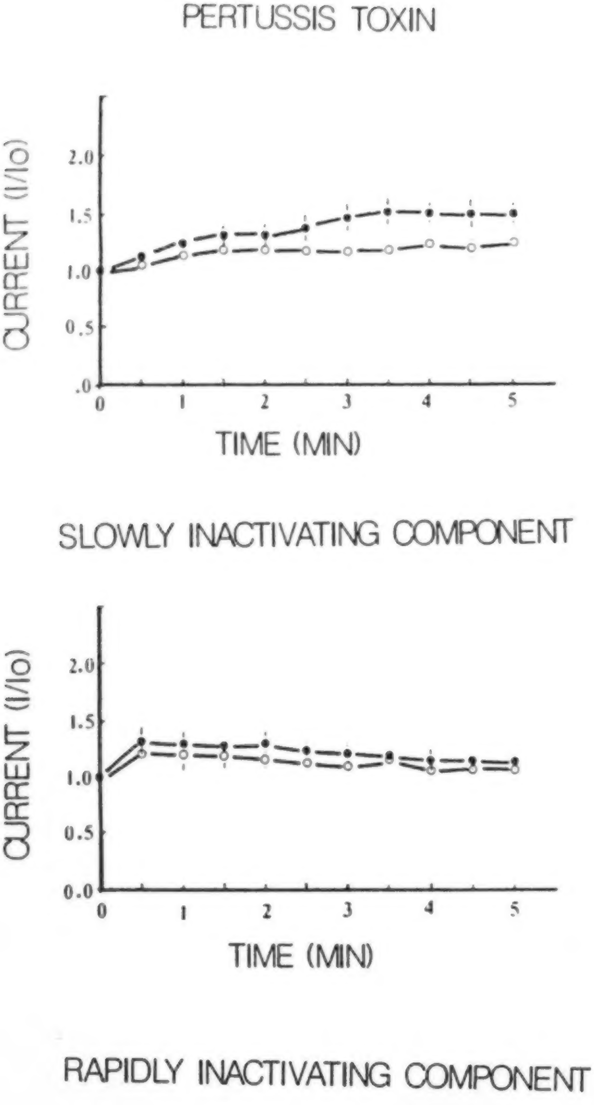


Fig. III-9. Comparison of groups of cells pretreated with pertussis toxin (PTX). The data are expressed in the same manner as described in Fig. 5. Pertussis toxin abolished the GTPγS-induced increase in Ca^{2+} channel currents (both rapidly and slowly inactivating Ca^{2+} channel currents) (n=6).

3.2. Neuroblastoma Cells

In the N1E-115 neuroblastoma cell line, under determined conditions, the cells express predominantly T channels (Wang *et al.*, 1990; Liu *et al.*, 1991). Thus, this preparation is a good model in which the effect of GTPγS on T channels was studied.

3.2.1. *Characterization of the T-Type Calcium Channel Current*

In a typical voltage-clamp experiment, the cells were depolarized using 200 ms voltage steps from a holding potential of -80 mV. The original current records (leakage corrected) and the corresponding current-voltage relationship are shown in Fig. III-10. The channel was activated at a potential of approximately -50 mV and inactivated completely within the 200 ms duration of the pulse. The amplitude of peak current occurred at a test potential of about -10 mV. This transient inward calcium channel current represented a typical T channel current (Narahashi *et al.*, 1987).

3.2.2 *The Effect of GTPγS on the T-Type Ca^{2+} Channel Current in N1E-115 Cells*

Fig. III-11 shows the original current records from two N1E-115 cells at 0, 5, 10 and 20 minutes after the control currents were established (see Methods section 4.1). The T channel current was activated by depolarizing the cell to -10 mV from a holding potential of -80 mV. The intracellular application of GTPγS did not show any effect on the T-type Ca^{2+} channel current in N1E-115 cells. The control cell showed a very small amount of current run-down during the 20 minute experiment, while the cell dialysed with GTPγS (500 μM) showed little change in inward current. In order to confirm this result, two groups of cells were compared with and without GTPγS in the pipette. Fig. III-12 shows the results of these experiments. The data were analyzed as described in the Methods section (4.1). GTPγS did not significantly increase or decrease the T channel current in neuroblastoma cells. The group of cells treated with GTPγS showed some run-down after 10 minutes, but this was not significant when compared to the control group (n=13).

Fig. III-10.

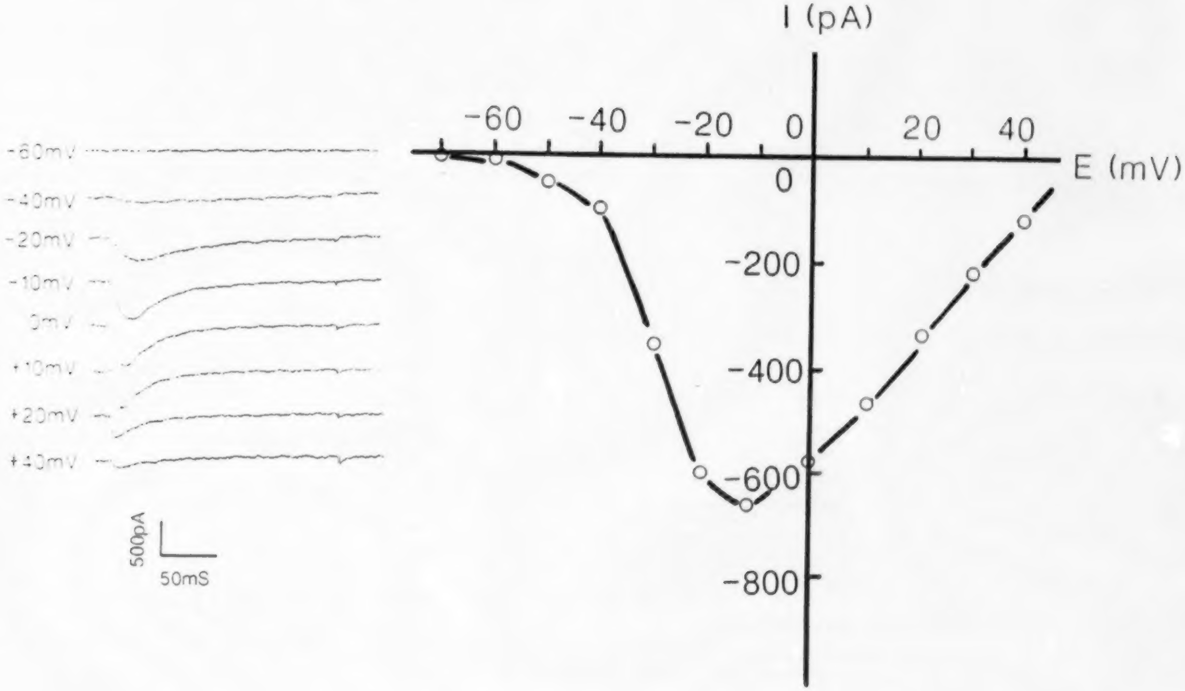


Fig. III-10. Original current records and the current-voltage relationship of the T channel current in a neuroblastoma cell (N1E-115). Current records shown on the left were evoked by a 200 ms test pulse from a holding potential of -80 mV to the test potential indicated next to each record. The I-V relationship (on the right) shows that the inward current was activated at -60 mV. The peak amplitude of this current occurred at approximately -10 mV. Current records were leakage corrected.

Fig. III-11.

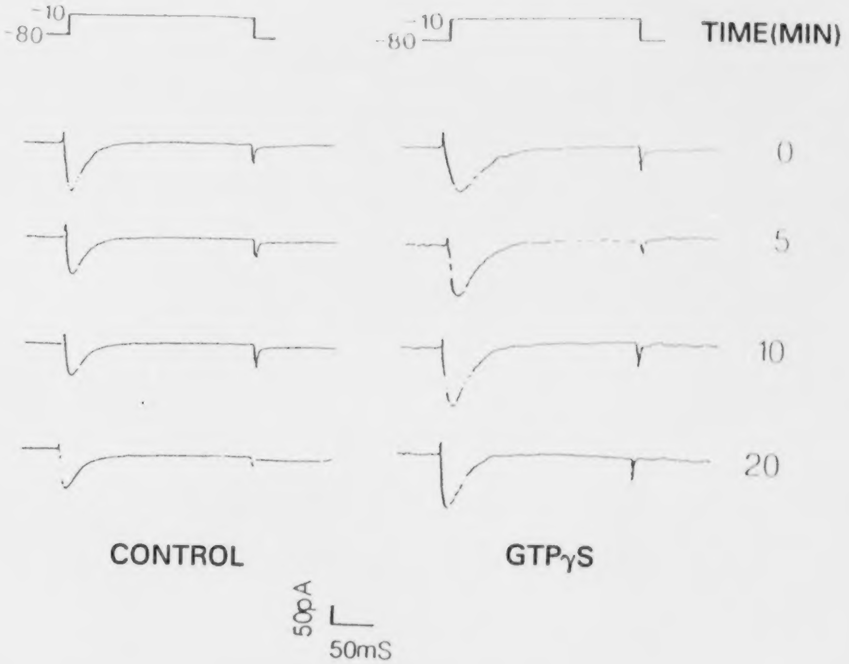


Fig. III-11. The current records from two neuroblastoma cells at 0, 5, 10 and 20 min after the establishment of the inward current. Currents were activated by depolarizing the cells to -10 mV from a holding potential of -80 mV. Intracellular application of 500 μM GTPγS did not have any effect on the Ca^{2+} channel current (T channel) in neuroblastoma cells. Current records were not leakage corrected.

Fig. III-12.

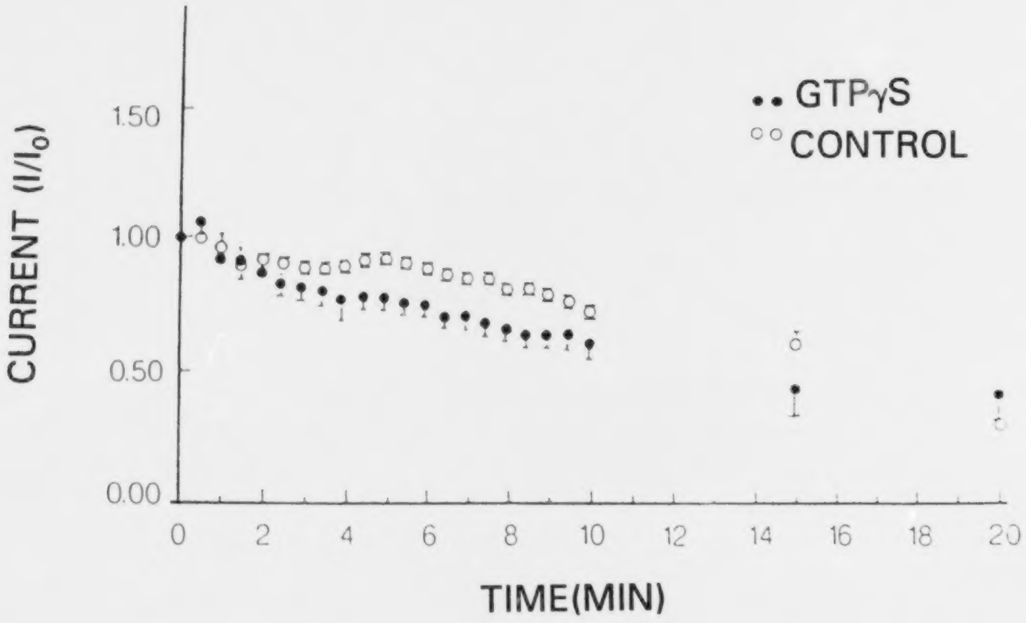


Fig. III-12. Comparison of the T channel currents in two groups of cells in the presence or absence of GTPγS in the pipette. The filled circles represent currents measured with GTPγS (500 μM) in the pipette and open circles represent the control currents. Current recordings (I) were obtained every 30 sec, compared with the initial current (I_0), and plotted against time. GTPγS did not significantly affect the T channel current in neuroblastoma cells. The values are given as the mean±S.E.M. Control: n=13, GTPγS: n=13. $^*P<0.05$

3.3. Vascular Smooth Muscle Cells (VSMC)

3.3.1. *Characterization of Ca^{2+} Channel Currents in VSMC (Rat Tail Artery)*

The existence of two types of calcium channels has been demonstrated using dissociated vascular smooth muscle cells (Bean *et al.*, 1986; Benham *et al.*, 1987a; Yatani *et al.*, 1987c; Nakazawa *et al.*, 1988). They are characterized as L- and T-type Ca^{2+} channels. Wang *et al.* (1989), working in this laboratory, first reported the two types of Ca^{2+} channel currents in VSMC from rat tail artery. Since rat tail artery is quite sensitive to many hormones and drugs, dissociated single cells from rat tail artery were used in these experiments. Fig. III-13 shows an inward current with a large T channel current component. On the left, the original current records are shown (holding potential of -80 mV). Depolarizing the cell to potentials between -30 and 0 mV elicited a time-dependent inward current. At more positive test potentials the current was small and sustained (did not inactivate within 200 ms), suggesting that another current component might also be present but that the current density was low. The I-V relationship plotted from the same cell showed that the channel was activated at -50 mV and the peak current occurred around -20 mV. Fig. III-14 shows an inward Ca^{2+} channel current which consists predominantly of the L channel component. Depolarizing the cell to various test potentials from a holding potential of -40 mV evoked the sustained current shown on the left of Fig. III-14. The currents did not inactivate during the 200 ms depolarization. The I-V relationship shows that the channel was activated at -20 mV and that the peak current occurred around +20 mV. This was consistent with the L-type Ca^{2+} channel current reported previously (Benham *et al.*, 1987a; Wang *et al.*, 1989).

3.3.2. *The Effect of GTPγS on the Inward Ca^{2+} Channel Currents in VSMC*

The effect of the intracellular application of GTPγS is shown in Fig III-15. GTPγS (500 μM) not only increased the L-type Ca^{2+} channel current but also shifted the peak current of the I-V relationship towards more negative potentials (by 10 mV). The increase was more obvious in the negative potential range. Fig. III-16 shows the current records from two cells, one without GTPγS (control) and one with 500 μM GTPγS in the pipette. The currents were elicited by depolarizing the cell to a test potential of -10 mV from a holding potential of -80 mV (leakage was not corrected). The effect of GTPγS on inward Ca^{2+} channel currents was observed as a function of time and compared to the control cell. Fig. III-17 shows the effect of GTPγS on the inward Ca^{2+} channel current in two groups of cells, one with GTPγS in the pipette and one without (control). GTPγS significantly increased the inward Ca^{2+} channel currents in vascular smooth muscle cells. This is in agreement with the report by Zeng *et al.* (1989a; b). The currents were recorded every minute and then compared with the initial current (I_0) plotted against time (see Methods section 4.1). These results revealed that the maximal effect of GTPγS on Ca^{2+} channel currents occurred at 2 minutes after the initial current was established. This increased effect of GTPγS on Ca^{2+} channel current was maintained for up to 10 minutes and then the current declined. The reason for the decline is uncertain. It could be due to Ca^{2+} channel current run-down or an inhibitory effect of GTPγS.

Fig. III-13.

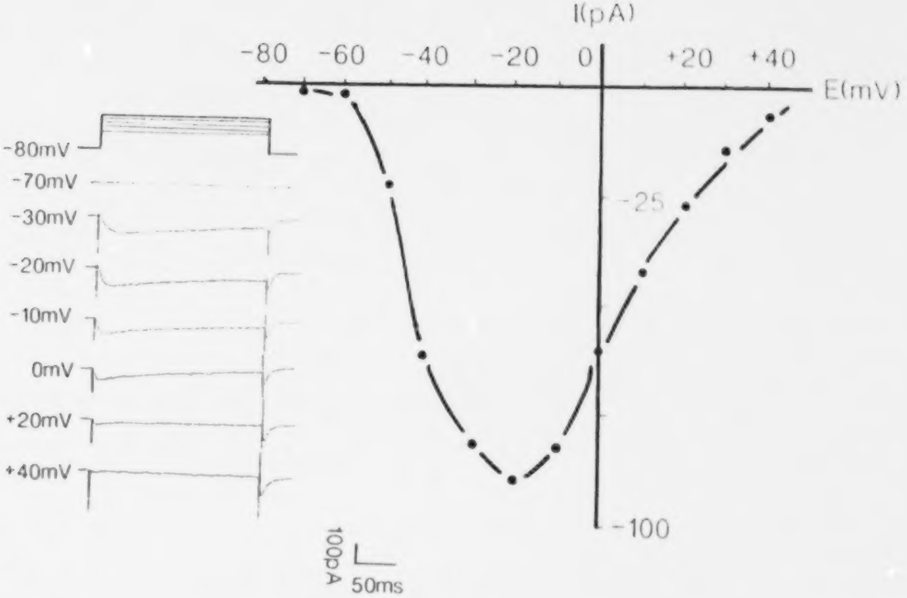


Fig. III-13. Original current records and the I-V relationship of Ca^{2+} channel currents in a vascular smooth muscle cell (primary culture). Current records shown on the left were evoked by a 250 ms test pulse from a holding potential of -80 mV to various test potentials which are indicated next to each record. The current records showed some inactivation within 50 ms. The I-V relationship (on the right) shows an inward current which was activated at -50 mV. The peak amplitude occurred at approximately -20 mV (leakage corrected). This cell expressed predominantly the T channel current component with only a small L channel current component.

Fig. III-14.

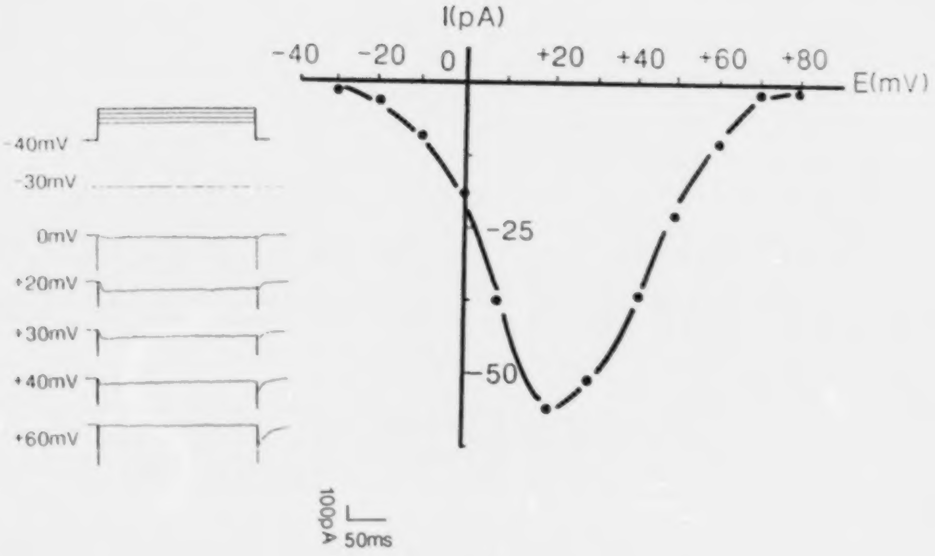


Fig. III-14. Original current records and the I-V relationship of the L-type Ca^{2+} channel current. Current records shown on the left were evoked by a 200 ms test pulse from a holding potential of -40 mV to various test potentials as indicated next to the current records. The records did not show inactivation during the 200 ms depolarization. The I-V relationship (on the right) showed an inward current which was activated at -20 mV and the peak current occurred at +20 mV (leakage corrected).

Fig. III-15.

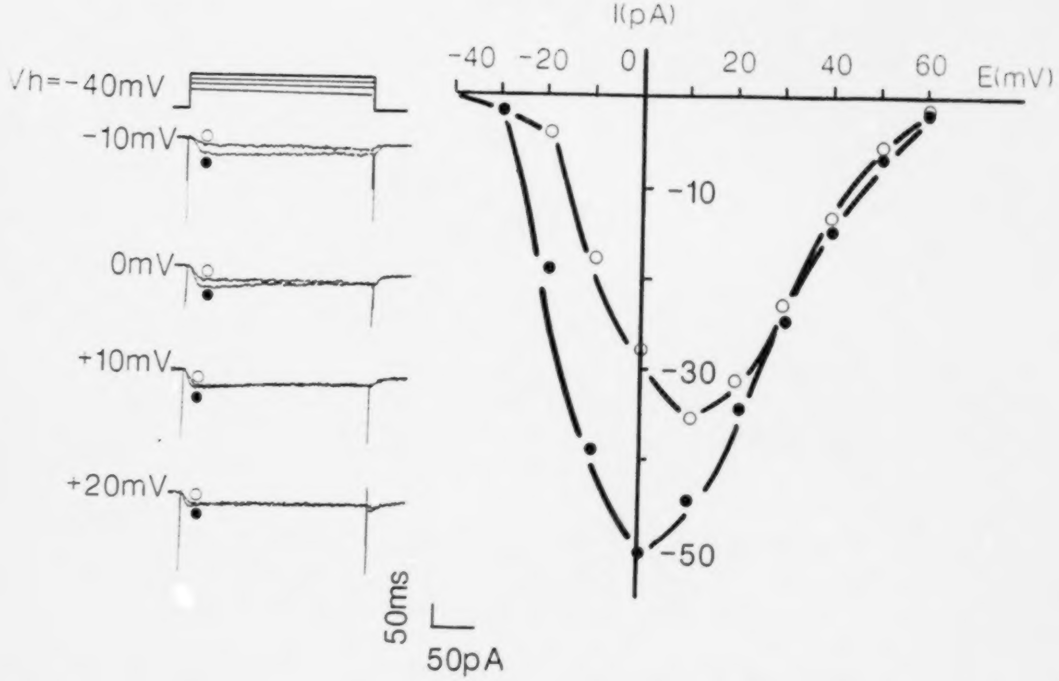


Fig. III-15. The effect of the intracellular application of GTPγS on the L-type Ca^{2+} channel current in a vascular smooth muscle cell. The filled circles represent GTPγS in the pipette and open circles represent the control current (initial current). GTPγS not only increased the L-type Ca^{2+} channel current but also shifted the peak current of the I-V relationship towards more negative potentials (about 10 mV).

Fig. III-16.

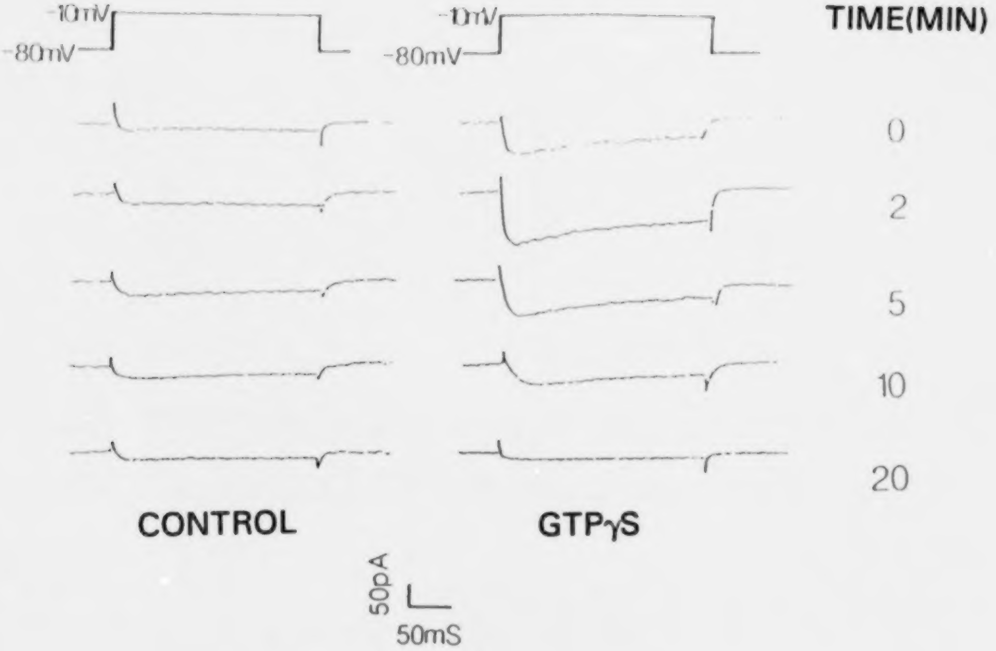


Fig. III-16. The current records from two cells, one without GTPγS in the pipette (control) and one with GTPγS in the pipette. The currents were activated by depolarizing the cell to -10 mV from a holding potential of -80 mV. The effect of GTPγS on inward Ca^{2+} channel currents was observed as a function of time. The effect on Ca^{2+} channel current (increase) reached its maximum 2 min after the inward current was established. After 10 min, the current declined (leakage was not corrected).

Fig. III-17.

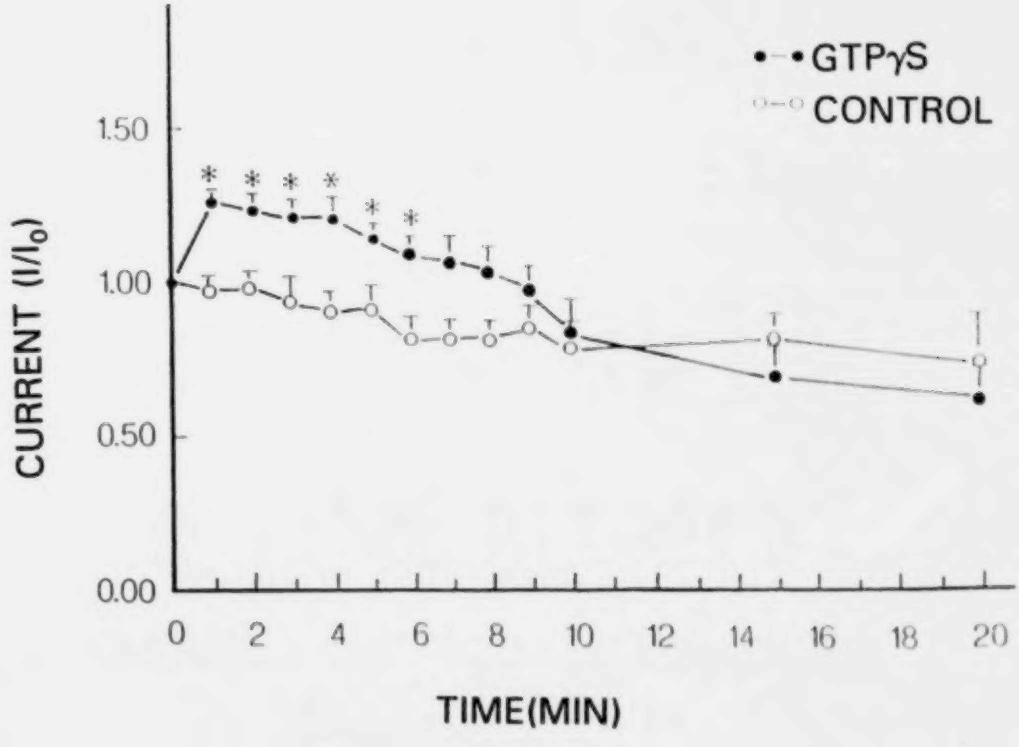


Fig. III-17. Comparison of two groups of cells in the presence or absence of GTPγS in the pipette. The filled circles represent cells treated with GTPγS (500 μM) and open circles represent control cells. Current recordings (I) were made every 30 sec, compared with the initial current (I_0), and plotted against time. GTPγS significantly increased the inward Ca^{2+} channel currents in vascular smooth muscle cells with time. The values are given as the mean±S.E.M. Control: n=9, GTPγS: n=9. $*P<0.05$

3.4. Neonatal Rat Ventricular Cells

3.4.1. *Characterization of Calcium Channel Currents in Ventricular Cells (Neonatal Rat)*

Both T- and L-type calcium channels have been identified in neonatal rat ventricular cells (Rampe *et al.*, 1989; Wang *et al.*, 1991a). From a holding potential of -80 mV, a depolarizing pulse to a test potential of 0 mV evoked a transient inward current which inactivated completely within the 150 ms test pulse duration (T-type calcium channel current) and a sustained component which inactivated slowly (L-type calcium channel current). The transient current (T) was activated at a membrane potential of -50 mV and the maximum amplitude occurred at about -10 mV (data not shown). The sustained current (L) activated at more positive potentials and the maximum current occurred at a membrane potential more positive than 0 mV, usually at +10 mV or +20 mV. Steady-state inactivation experiments have shown that when the holding potential is -40 mV, at least 90% of the T channel current is inactivated, which is in agreement with a previous report (Bean, 1989). Furthermore, the T channel was sensitive to Ni^{2+} (40 μM) (Fig. III-18A) and at this concentration, the L channel current was not affected (Fig. III-18B). This is consistent with the results obtained by others (Bean, 1989). T channels are not very sensitive to dihydropyridines (Bean, 1989) whereas the L-type channels are usually sensitive to dihydropyridines (Brown *et al.*, 1986). Bay K 8644 amplified the L channel current (Fig. III-18B). A high concentration of nifedipine was applied to the cells in order to completely block the L channel current (Fig. III-18C). The non-specific Ca^{2+} channel antagonist La^{3+} at a concentration of 2 mM totally blocked the L (Fig. III-18B) and T channel current (data not shown), confirming that these were Ba^{2+} currents carried by Ca^{2+} channels. Therefore, both T- and L-type channel currents could be measured in primary cultured neonatal rat ventricular cells and the two components of the macroscopic Ca^{2+} channel currents could be separated by using different holding potentials and by pretreatment with nifedipine.

It has been reported that in cardiac cell types, most of the macroscopic Ca^{2+} channel current was carried by the L channel and that the maximum T current amplitude was usually 5-20% of the total current. Some cell types appeared to have no T current at all (Bean, 1989). However, in this preparation of neonatal ventricular cells, the magnitude of the T current varied, ranging between 10-50% of the total maximum current (Wang *et al.*, 1991). Therefore, it was possible to investigate the effect of GTPγS on both T and L channel currents in these cells.

3.4.2. *Effects of GTPγS on Inward Ca^{2+} Channel Currents*

The intracellular application of GTPγS (500 μM) produced an increase in the amplitude of the inward Ca^{2+} channel currents at various test potentials. Fig. III-19 shows that GTPγS increased the L-type Ca^{2+} channel current in a ventricular cell. The effect of GTPγS on the Ca^{2+} channel current was determined by measuring the magnitude of currents (I_5) 5 minutes after the initial current (I_0) was established (see Methods section 4.1). Fig. III-19A shows the original current records from a ventricular cell. The cell was depolarized from a holding potential of -40 mV to various potentials as indicated next to the current records. GTPγS (filled circles) increased the L-type Ca^{2+}

channel current when compared with the initial current (open circles). Fig. III-19B shows the I-V relationship from the same cell shown in Fig. III-19A. GTPγS not only increased the amplitudes of the L-type Ca^{2+} channel current but also shifted the peak current of the I-V relationship towards more negative potentials (10 mV). The effect of GTPγS on the Ca^{2+} channel current was more prominent at negative potentials. Similar effects of GTPγS were observed in cells which were depolarized from a holding potential of -80 mV using the same protocol described above. Fig. III-20 shows that GTPγS increased the total inward Ca^{2+} channel current (both T- and L-type current components). Fig. III-20A shows the original current records from a cell depolarized from a holding potential of -80 mV to various test potentials (leakage corrected) and Fig. III-20B shows that GTPγS increased total inward Ca^{2+} channel currents (I-V relationship). GTPγS not only increased total inward Ca^{2+} channel currents, but also shifted the peak current towards more negative potentials as shown in Fig. III-19B.

Since the effect of GTPγS on Ca^{2+} channel currents in these experiments was a function of the time, the magnitudes of the currents were measured as a function of time after the initial current was established. Fig. III-21 shows the comparison of the current amplitudes as a function of time in two groups of cells. One group of cells was dialysed with GTPγS and the other group of cells was dialysed with normal pipette solution (control). The currents were activated by depolarizing the cells from a holding potential of -40 mV to a test potential of +10 mV. Current records from two representative cells are shown in Fig. III-21A. The current amplitude 5 minutes after the initial current did not change much in the cell dialysed with normal solution. However, in the presence of intracellular GTPγS, the current amplitude at 5 minutes increased when compared to the initial current. In order to confirm this effect, these experiments were repeated in groups of cells and the data is shown in Fig. III-21B. Filled columns represent the control values (in the absence of GTPγS) (n=8) and the dotted columns represent the presence of GTPγS (n=8). GTPγS increased the L-type Ca^{2+} channel current in neonatal ventricular cells. The current amplitudes as a function of time were measured and normalized to the initial current (I/I_0), and then plotted against time as shown in Fig. III-21B. The current amplitudes was also increased at 3 minutes in the control group. This may be due to incomplete dialysis of the cell and the fact that outward K^+ was not totally blocked by Cs^+ inside the pipette. The same protocol was used to measure the effect of GTPγS on the total inward Ca^{2+} channel current (Fig. III-22). Fig. III-22A shows the original current records from two cells. The currents were activated by depolarizing the cells from a holding potential of -80 mV to a test potential of 0 mV. Fig. III-22B shows the current amplitudes from two groups of cells, one in the absence of GTPγS (black columns) (n=7) and one with GTPγS (dotted columns) (n=10). GTPγS also increased the total inward Ca^{2+} channel currents in neonatal rat ventricular cells. Fig. III-23 shows that the intracellular application of GTPγS increased the total inward Ca^{2+} channel current and that the increase was concentration dependent. The currents were measured 5 minutes after the initial current was established. The holding potential was -80 mV and the currents were activated by depolarizing the cell to -10 mV. The current at 5 minutes was normalized to the initial current. Different concentrations of GTPγS were applied intracellularly and the normalized current was plotted against the intracellular concentration of GTPγS.

3.4.3. *Effect of GTPγS on the Steady-State Activation and Inactivation of L-Type Ca^{2+} Channel Currents*

The effect of GTPγS on the steady-state activation curve is shown in Fig. III-24. The current amplitudes were measured 5 minutes after the initial current was established. Tail currents were measured and normalized to the maximal current which was obtained at the test potential that gave the largest tail current. The ratio of the resulting tail currents (I_{test}/I_{max}) was determined and plotted against the V_{test} in Fig. III-24. The membrane potential was held at -60 mV and a 50 ms test pulse to various potentials was applied as indicated in the inset of the figure. The Boltzmann equation $I=I_{max}[1+\exp(V-V_h)/k]^{-1}$ was used to fit the experimental data. In this equation, V represents the holding potential, V_h is the potential at which one half of the Ca^{2+} channels are activated and k is the slope factor. The results show that the intracellular application of GTPγS shifted the steady-state activation curve towards more negative potentials by about 10 mV. V_h(control) $=12.3$ mV, V_h(GTPγS)$=1.6$ mV. The slope of the two curves as determined from the curve fit were similar ($k_1=11.6$; $k_2=11$). This experiment was repeated five times and similar results were obtained.

Experiments described previously showed that GTPγS not only increased the inward Ca^{2+} channel currents but also shifted the peak current of the I-V relationship towards more negative potentials. To determine whether or not the GTPγS effect was membrane potential dependent, steady-state inactivation curves were measured using the current amplitude at 5 minutes (I_5) after the initial current was established (Fig. III-25). The membrane potential was adjusted to the different conditioning potentials before the application of a test pulse to +20 mV. Current amplitude measurements were normalized to the value obtained with the most negative potential under each experimental condition. In the case of the control cells, the currents were reduced in amplitude at the more depolarized conditioning potentials and were completely inactivated at +5 mV. GTPγS shifted the steady-state inactivation curve of the Ca^{2+} channel current (L-type) curve towards more negative potentials by 7 mV. The protocol used in this experiment is displayed in the inset of Fig. III-25. The experimental data was also fitted by the Boltzmann equation as described above. V_h(control)=-7.2 mV, V_h(GTPγS)=-15 mV. The slope factors of the two curves were k_1=-4.6 and k_2=-5.5. The steady-state inactivation experiment was repeated six times.

3.4.4. *Comparison of Total Inward Ca^{2+} Channel Currents and L-Type Ca^{2+} Channel Currents*

The effect of GTPγS on the total inward Ca^{2+} channel current (HP=-80 mV) and L-type Ca^{2+} channel current (HP=-40 mV) was investigated. The effect of GTPγS on the total current was increased about 80.5%±16.9, and about 46.4%±7.2 (percentage of increase) on the L-type current when compared with the control (Fig. III-26), indicating that another type of Ca^{2+} channel current could be also increased by GTPγS. It has been demonstrated in Fig. III-18 that the neonatal rat ventricular cell preparation contained two Ca^{2+} channel current components, the L and T channel current. Thus, the results shown in Fig. III-26 suggest that GTPγS may also increase T channel current in this preparation. Since this was measured as percentage of increase, it is assumed that the T channel current might be more sensitive to GTPγS than was the L current. However, at this stage, there is not sufficient evidence to draw a concrete conclusion. More experiments are needed to demonstrate that the T channel current can also be modulated by GTPγS.

3.4.5. ***Effect of Caged GTPγS on Ca^{2+} Channel Current***

Since the initial current in the experiment described above varies possibly due to incomplete dialysis and K^+ inhibition, flash photolysis experiments were performed. Caged-GTPγS was included in the patch pipette, so that once it diffused into the cell it could be activated rapidly by photolysis, yielding free GTPγS (Dolphin *et al.*, 1988). Caged-GTPγS was used to determine the feasibility of using the initial current as the control value in the experiments described in previous studies. The results from this experiment (Fig. III-27) show that release of GTPγS by flash photolysis increased the inward Ca^{2+} channel current (L-type) as was observed previously. It has been reported that a complete dialysis of the intracellular constituents requires 5 minutes after rupture of the membrane (Doroshenko *et al.*, 1982). The time depends on the pipette diameter and cell volume (Pollo *et al.*, 1991). When the inward current was established and stabilized for 5-10 minutes, flash photolysis (see Methods section 4.2) was used to release caged-GTPγS (100 μM) which had diffused into the cell interior. The experiment was repeated four times and similar results were obtained. With this technique, a control current was established. This result supported the previous observation that the intracellular application of GTPγS increased Ca^{2+} channel currents and confirmed that the use of the initial current as a control was acceptable.

Fig. III-18.

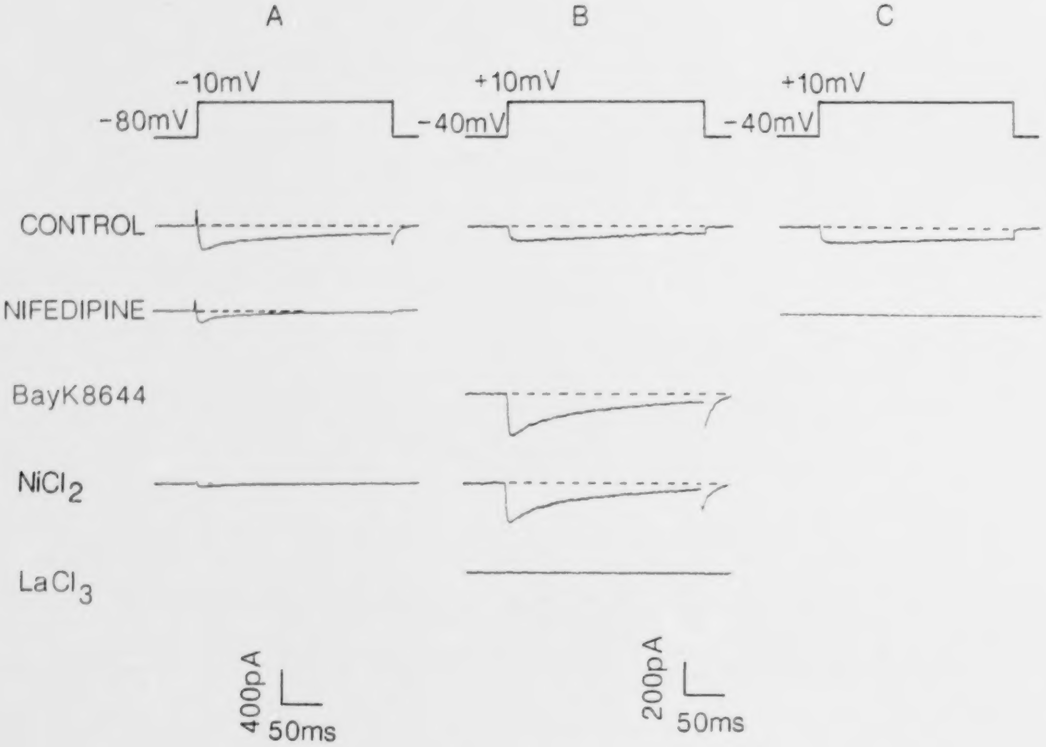


Fig. III-18. Original current records (leakage corrected) from three neonatal rat ventricular cells. A. A test pulse from a holding potential of -80 mV to -10 mV produced an inward current consisting of two components. One component inactivated rapidly (T-type) and the other inactivated slowly (L-type). Nifedipine (10^{-5} M) blocked the L and did not affect the T channel current. The T current was almost completely inhibited by 40 μM Ni^{2+}. B. A test pulse to +10 mV from a holding potential of -40 mV primarily activated the L channel current, since the T current was almost completely (90%) inactivated. Bay K 8644 (1 μM) increased this current, whereas Ni^{2+} (40 μM) did not affect the L-type Ca^{2+} channel current. However, the L channel current could be blocked by 2 mM La^{3+}. C. A test pulse to +10 mV from a holding potential of -40 mV evoked an L channel current as shown in B and this was completely blocked by 10 μM nifedipine. The results shown in A, B and C are from 3 different cells and the drugs were added into the bath sequentially. The calibration bars are the same for B and C.

Fig. III-19.

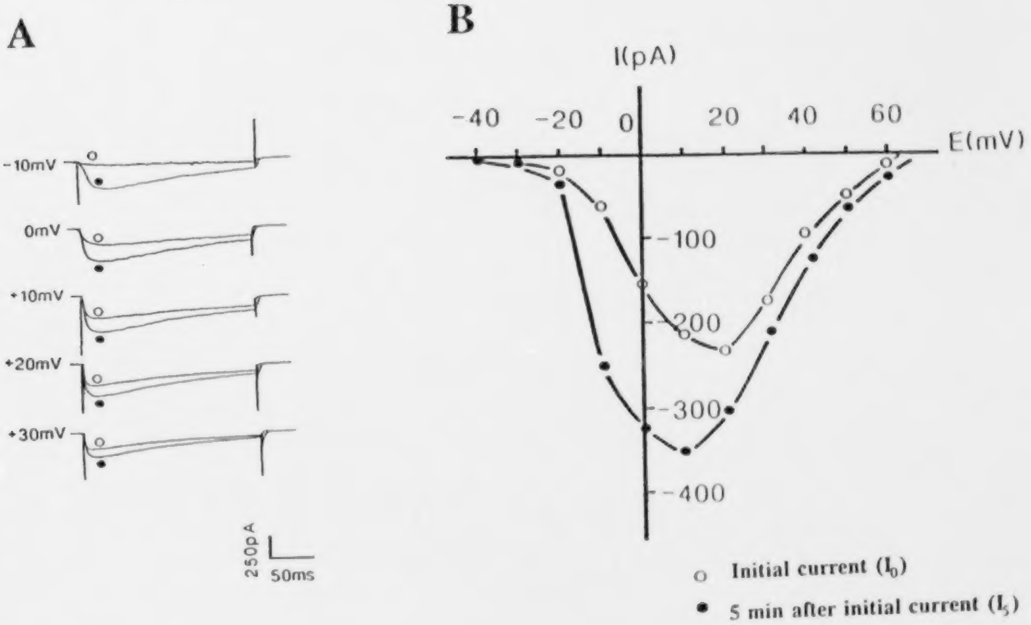


Fig. III-19. Effect of GTPγS (500 μM) on the L-type Ca^{2+} channel current. A. Original currents were activated by the test potentials indicated next to each record (holding potential of -40 mV). GTPγS (5 min after the initial current, filled circles) increased L-type Ca^{2+} current when compared with control values (initial current, open circles). B. The I-V relationship of the L channel current before and 5 min after the initial current in a GTPγS dialysed cell. GTPγS not only increased Ca^{2+} channel currents, but also shifted the I-V relationship towards more negative potentials (about 10 mV). The currents shown in A are plotted in B. Original records were leakage corrected.

Fig. III-20.

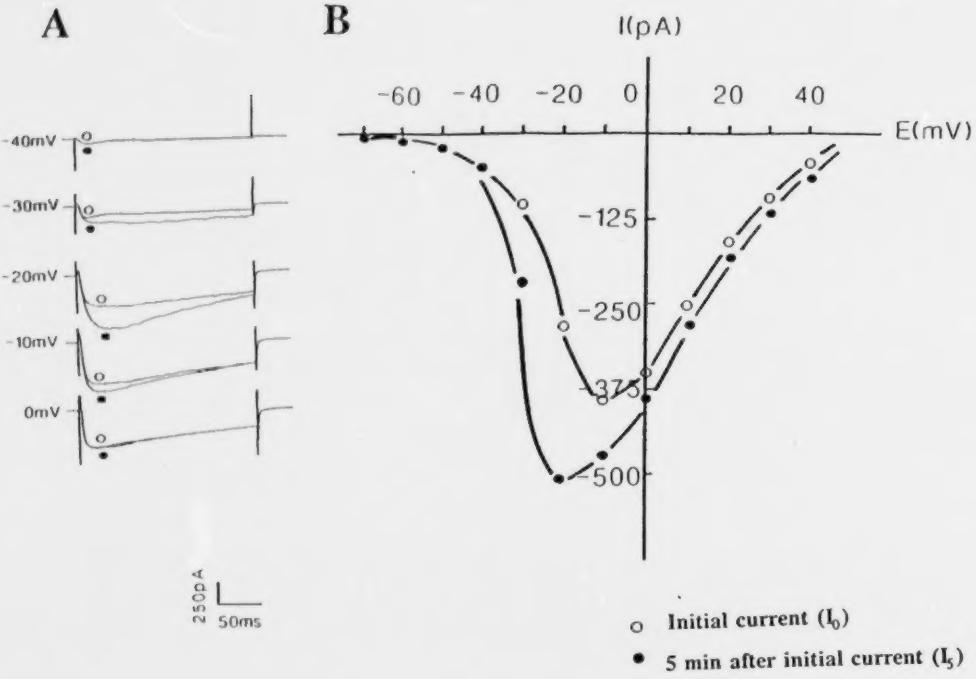


Fig. III-20. Effect of GTPγS (500 μM) on the total Ca^{2+} channel current. A. Original current records activated by the test potentials which are indicated next to each record (holding potential of -80 mV). GTPγS (5 min after the initial current, filled circles) increased total Ca^{2+} current when compared with control values (initial current, open circles). B. The I-V relationship of the total Ca^{2+} channel current before and 5 min after the initial current in a GTPγS dialysed cell. GTPγS not only increased Ca^{2+} channel current, but also shifted the I-V relationship towards more negative potentials (about 10 mV). The currents shown in A are plotted in B. Original records were leakage corrected.

Fig. III-21.

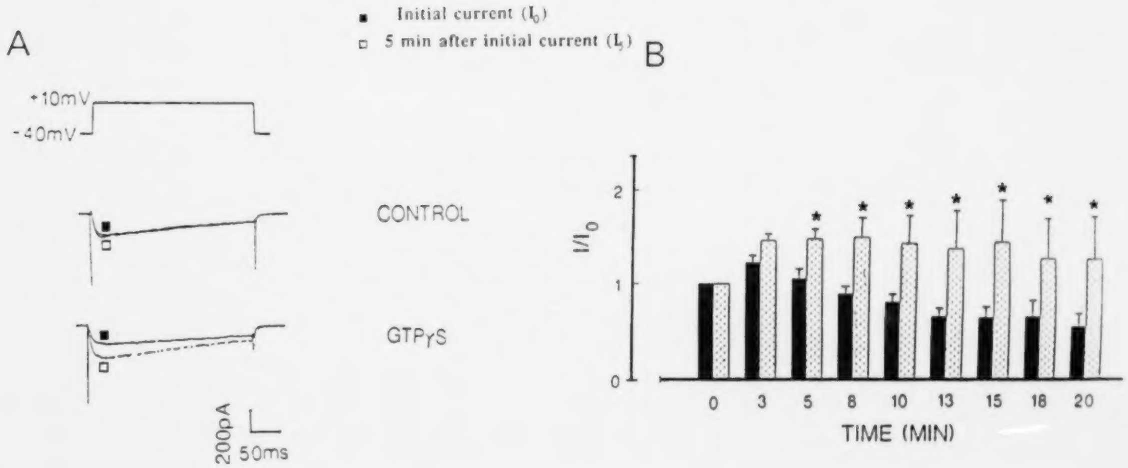


Fig. III-21. Effect of GTPγS on the L channel current as a function of time. A. The effect of GTPγS on the L channel current was compared in two cells. Upper traces show the original current records in the cell without intracellular GTPγS. Lower traces show the current records in the cell dialysed with GTPγS. Filled squares represent initial current (control) and open squares represent current 5 min after initial current (GTPγS) (Methods section 4.1). B. The effect of GTPγS on L-type Ca^{2+} channel current was obtained at different times as indicated in the figure. The current amplitudes were compared with the initial current (I/I_0) and plotted against time. GTPγS significantly increased the L channel current. The current in the control group showed an initial increase and subsequently some run-down. $^*P<0.05$.

Fig. III-22.

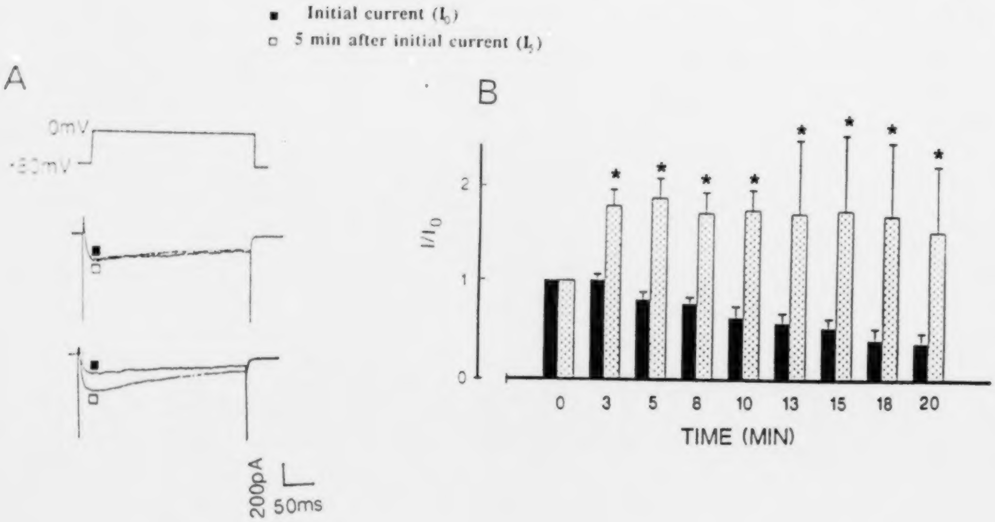


Fig. III-22. Effect of GTPγS on total inward currents as a function of time. A. The effect of GTPγS on the total Ca^{2+} channel current in a control cell (upper records) and GTPγS dialyzed cell (lower records). Filled squares represent initial current (control) and open squares represent current after administration of GTPγS. B. The effect of GTPγS on total Ca^{2+} channel current at different times in two groups of cells as indicated in the figure. The current amplitudes were compared with initial current and plotted against time. GTPγS significantly increased total Ca^{2+} channel current while the control cells showed substantial run-down. $^*P<0.05$

Fig. III-23.

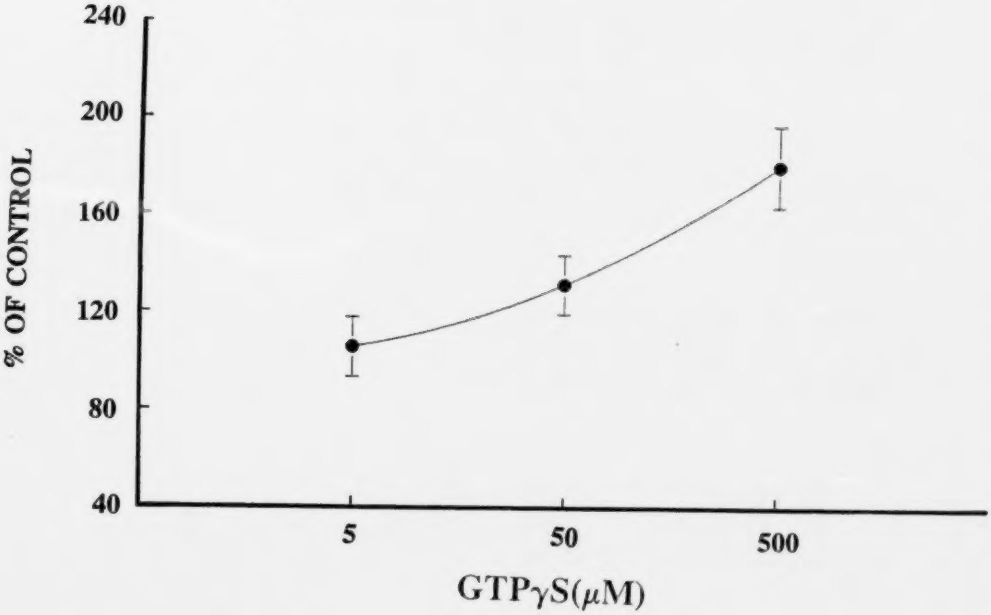


Fig. III-23. The effect of different concentrations of GTPγS on the total Ca^{2+} channel currents (HP=-80 mV). Different concentrations of GTPγS (50-500 μM) were applied intracellularly and the currents were measured 5 min after initial current. The data are expressed as mean±S.E.M. (n=3).

Fig. III-24.

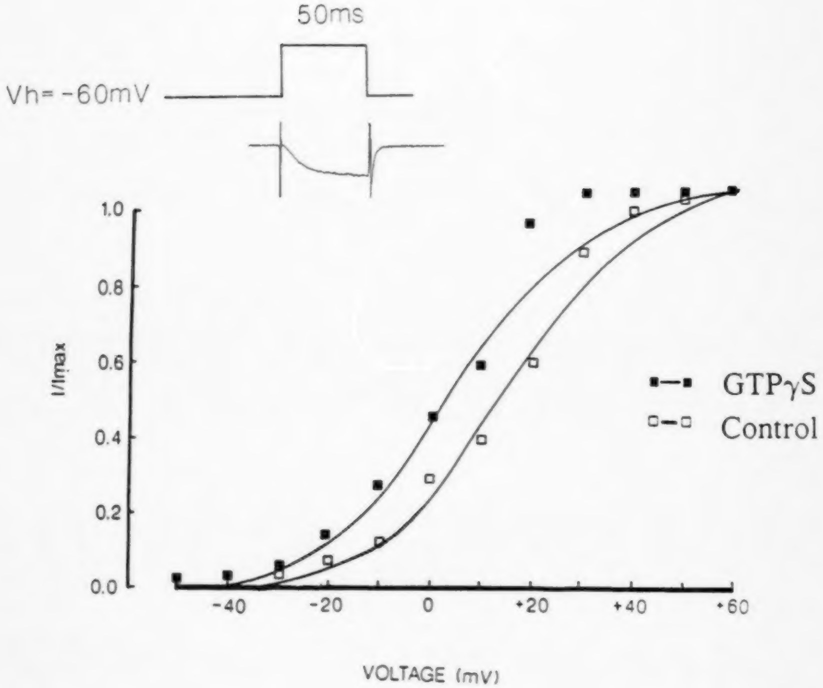


Fig. III-24. The effect of GTPγS on the steady-state activation of the L-type Ca^{2+} channel current. The magnitudes of I_{max} and I were determined from the tail currents as shown in the inset. GTPγS shifted the steady-state activation towards more negative potentials. Curves were fitted as described in the Methods section (7).

Fig. III-25.

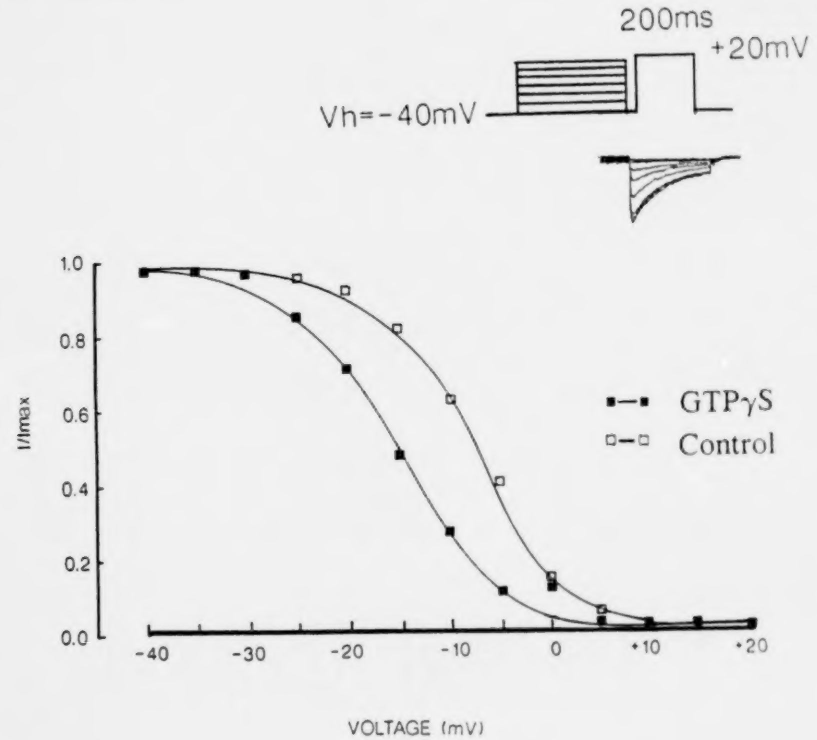


Fig. III-25. The effect of GTPγS on the steady-state inactivation of the L-type Ca^{2+} channel current. The membrane was clamped at various conditioning potentials for 1 sec and followed by a test pulse to +20 mV for 200 ms. The amplitudes of the inward current evoked by the test pulse were normalized to the maximal current. The protocol is shown in the inset of Fig. III-25. GTPγS shifted the steady-state inactivation curve towards more negative potentials. Curves were fitted as described in the Methods section (7).

Fig. III-26.

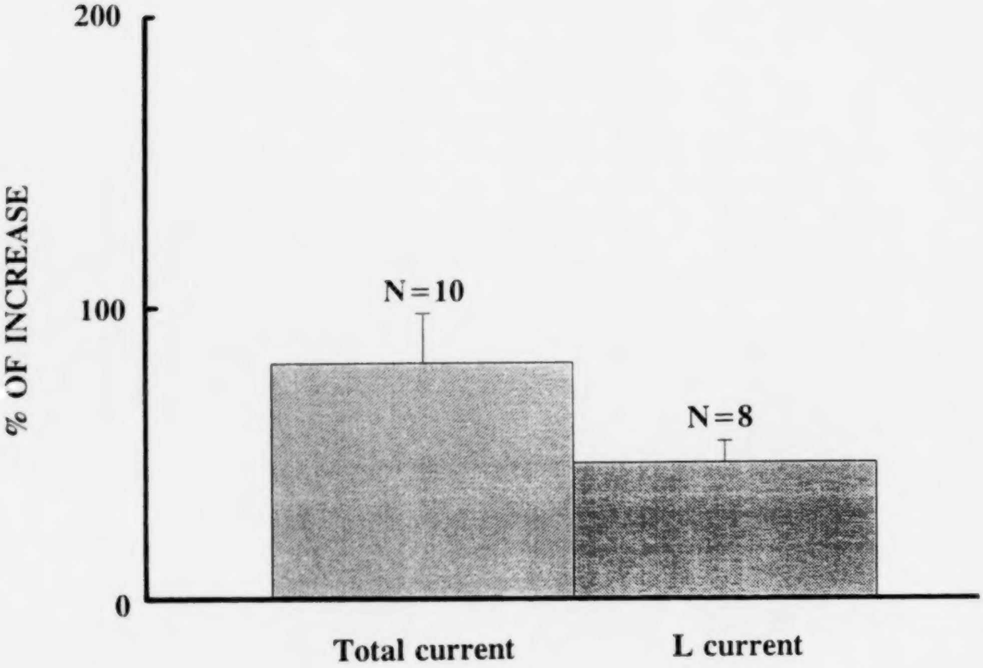


Fig. III-26. Comparison of the L-type and total Ca^{2+} channel current increase induced by GTPγS (500 μM). The current was measured 5 min after the initial current (I_0) and normalized to I_0. The total increase induced by GTPγS was larger than the L-type current increase, suggesting that GTPγS might also increase the T-type Ca^{2+} channel current. The histogram was constructed using I_0 as 100%. Currents greater than I_0 were plotted as percentage of increase. The percentage increase was calculated as follows: ($I/I_0 \times 100\% - 100\%$).

Fig. III-27.

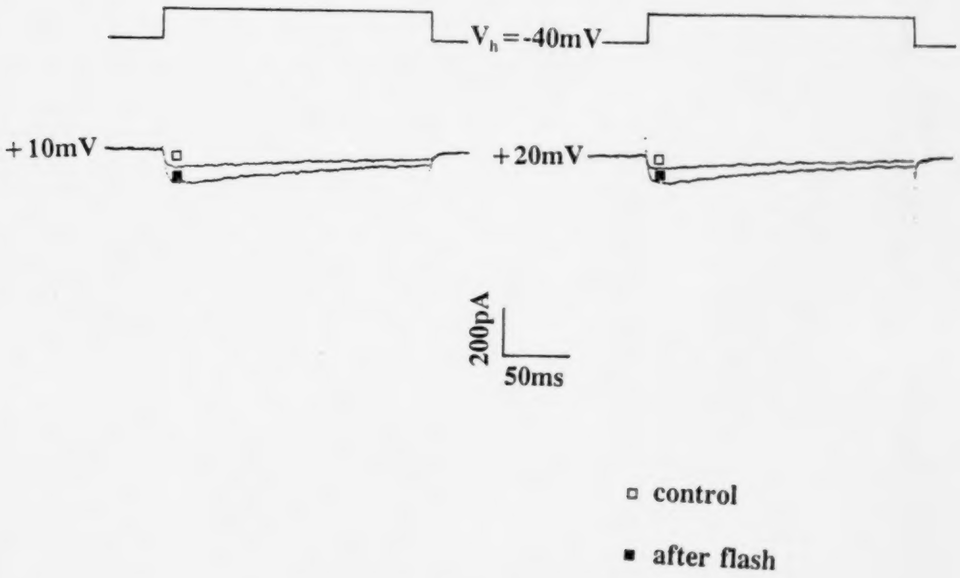


Fig. III-27. The effect of caged-GTPγS on Ca^{2+} channel current (L-type). Caged-GTPγS (100 μM) was applied intracellularly. The holding potential was set at -40 mV and the cell was depolarized to +10 mV and 20 mV. Two 148 J, 1 ms UV pulses were applied as described in the Methods section (4.2). The current records at two test potentials are shown before (open squares) and after the flash (filled squares). GTPγS increased the L-type Ca^{2+} channel current.

4. Discussion

4.1. GH$_3$ Cells

The data demonstrate that GH$_3$ cells expressed two types of voltage-dependent Ca^{2+} channel currents, a rapidly and a slowly inactivating component. This has been reported in previous studies (Armstrong & Matteson, 1986). Intracellular application of GTPγS produced significant effects on these two types of Ca^{2+} channel currents. These effects are not due to an increase in K$^+$ channel currents (Yatani *et al.*, 1987b), since the K$^+$ channel blockers Cs$^+$ and TEA$^+$ were used in these experiments. In addition, the effect of GTPγS was compared with a control group (in the absence of GTPγS) under identical experimental conditions.

GTPγS binds to the G protein α-subunit to form a complex which may modulate Ca^{2+} channels directly (Brown & Birnbaumer, 1988). Alternatively, the complex may induce a conformational change in the Ca^{2+} channel via intracellular second messengers. Studies have shown that cAMP-dependent phosphorylation (Armstrong & Eckert, 1987), protein kinase C (Hofmann *et al.*, 1987) and diacylylglycerol (Michel *et al.*, 1988) may be involved in the modulation of the voltage-dependent Ca^{2+} channels. The data presented here show that in GH$_3$ cells, GTPγS affected both the rapidly and slowly inactivating current components, initially increasing, and subsequently decreasing, these currents. One possible interpretation for this time-dependent increase in Ca^{2+} currents is that the internal pipette solution containing GTPγS gradually activated G proteins on the inner side of the membrane due to diffusion restrictions. GTPγS diffused into the cell and G protein activation resulting from GTPγS binding to α-subunits was limited by the relatively small diameter of the pipette and the large size of the cells. Therefore, it takes time for GTPγS to activate the G proteins (Hescheler *et al.*, 1987b). However, the mechanism underlying G protein activation of Ca^{2+} channels needs to be explored further with respect to the mechanism of action (direct activation or via a second messenger pathway).

The present studies suggest that multiple G proteins may modulate Ca^{2+} channels. The data presented here using cells pretreated with toxins support this suggestion. It was observed that PTX abolished the effects of GTPγS on the increase in Ca^{2+} channel currents. In support of this finding, a recent report has suggested that in GH_3 cells a PTX-sensitive G protein is also responsible for the potentiation of voltage-dependent Ca^{2+} channel current stimulated by LHRH (luteinizing hormone releasing hormone) (Rosenthal *et al.*, 1988a). However, in contrast, it has also been reported that in pituitary cells, a PTX-sensitive G protein mediates the inhibitory effect of hormones on voltage-dependent Ca^{2+} channels (Lewis *et al.*, 1986), which is inconsistent with the data obtained in this chapter. The 39 KDa (G_o) and the 41 KDa (G_i) proteins which can be ADP-ribosylated by PTX (Zysk *et al.*, 1986) have been identified in the GH_3 cell membrane. A high concentration of G_o is also reported to be present in GH_3 cells (Rosenthal *et al.*, 1988a,b). On the other hand, CTX abolished the effect of GTPγS on the subsequent decrease of Ca^{2+} channel currents, indicating that different G proteins may be involved in the regulation of Ca^{2+} channels. Although previous studies have suggested that the CTX-sensitive G protein was stimulatory, the inhibitory effect of a novel CTX-sensitive G protein (G_c) on receptor-mediated phosphoinositide signalling was reported in human pituitary cells (Low & Hughes, 1987). Nevertheless, our results clearly show that in GH_3 cells, a PTX-sensitive G protein may be responsible for the increase in Ca^{2+} channel currents and a CTX-sensitive G protein may be responsible for the subsequent decrease in Ca^{2+} channel currents.

Several different G proteins have been found in the pituitary cell membrane, a novel CTX-sensitive G protein (Low & Hughes, 1987), a PTX-insensitive G protein (Offermans *et al.*, 1989) and a G protein insensitive to both PTX and CTX (Matin *et al.*, 1986). In addition to modulation of VDCC in GH_3 cells, G proteins are involved in many intracellular actions, such as receptor-PIC (phosphoinositidase C) coupling and desensitization of the receptors (Simard & Labrie, 1986; Low & Hughes, 1987). Therefore, the G protein related signalling transduction mechanism can be a very complex system. Although the effects of GTPγS on Ca^{2+} channel currents are in fact the summation of all activated G proteins, our studies provide strong evidence that the direct stimulation of G protein independent of hormonal application regulates VDCC.

4.2. **Neuroblastoma Cells**

Neuroblastoma cells (N1E-115) cultured under the conditions described in the Methods section (1.2) expressed predominantly T-type Ca^{2+} channels. The steady-state inactivation experiments showed that at a holding potential of -40 mV, 95% of the current was inactivated. This T channel current was not sensitive to dihydropyridines (Liu *et al.*, 1991). The results from the experiments showed that the intracellular application of GTPγS did not significantly affect the T-type Ca^{2+} channel current in neuroblastoma cells. However, it has been shown that the intracellular application of GTPγS could cause inhibition or stimulation of the T channel current in neurons. For example, dopamine and adenosine analogues were found to inhibit T channel currents in chick DRG neurons (Marchetti *et al.*, 1986; Scott & Dolphin, 1987). Caged-GTPγS (10-20 μM) also inhibited the T channel current in DRG (Scott *et al.*, 1990). However, it has also been reported that low concentrations of GTPγS (6 μM) increased the T channel current in chick DRG neurons (Scott *et al.*, 1990). In bovine glomerulosa cells, angiotensin II stimulated T-type Ca^{2+} channel currents through a G protein pathway (Cohen *et al.*, 1988). However, in this study, neuroblastoma cells are a cell line derived from undifferentiated murine neuroblastomas. Since different cells have different G proteins and their coupling system to Ca^{2+} channels may also vary, it is difficult to extrapolate the effect of GTPγS on Ca^{2+} current from one cell type to another.

Since GTPγS activates all G proteins, the ultimate effects on the T channel reflect the summation of all G protein activation. In the present study, an effect of GTPγS on the T channel current was not observed. There are several possibilities: 1) GTPγS did not effectively diffuse into the cell. This is very unlikely, because the same methods were used in other cell types, and the effect of GTPγS on Ca^{2+} channel currents was observed. 2) GTPγS activated several G proteins and the summation of the activation (stimulation and inhibition) was no effect. This is also a likely possibility. However, at this point, this possibility cannot be completely excluded. 3) GTPγS did diffuse into the cell and activated G proteins. However, the activated G protein were not coupled to T channel proteins in neuroblastoma cells. This is also a likely possibility. 4) G proteins in these tumor cells may not be functional.

4.3. Vascular Smooth Muscle Cells from Rat Tail Artery

The data described in this study show that primary cultures of vascular smooth muscle cells expressed two types of voltage-dependent Ca^{2+} channels. One type activated by small depolarizations inactivated quickly (T-type channel) and the other type required a greater depolarization for activation and inactivated slowly (L-type channel) (Bean *et al.*, 1986). These two types have been previously identified in this preparation (Wang *et al.*, 1989). Since there are no Na^+ channels expressed in these vascular smooth muscle cells, the concentration of TTX used in the experiment was low. Using TEA^+ outside and Cs^+ inside the pipette, and Ba^{2+} as the charge carrier, the K^+ channels were blocked. Hence, an increase in Ca^{2+} channel current was not masked by an inhibition of outward K^+ currents.

The effect of GTPγS on inward Ca^{2+} channel currents was compared using two groups: the control group and a group with GTPγS in the pipette. The experimental conditions for the two groups were identical. The results from these experiments clearly demonstrate that GTPγS activated Ca^{2+} channel currents in vascular smooth muscle cells. This is consistent with the indirect evidence from previous reports (Zeng *et al.*, 1989a,b). GTPγS not only increased the current amplitudes of Ca^{2+} channel currents, but also shifted the peak current of the I-V relationship towards more negative potentials (5-10 mV). This is similar to the results obtained with ventricular cells. It has been reported that GTPγS-induced vasoconstriction was dependent on the concentration of GTPγS and also dependent on extracellular Ca^{2+} concentration (Zeng *et al.*, 1989b). Therefore, it was proposed that the contraction induced by GTPγS resulted in the elevation of cytosolic Ca^{2+} that was due to extracellular Ca^{2+} influx rather than Ca^{2+} release from intracellular Ca^{2+} pools. This observation was consistent with the results obtained in this section that GTPγS increased Ca^{2+} channel currents.

Although previous reports have shown that receptor activation resulted in Ca^{2+} channel activation or inactivation via G proteins in vascular smooth muscle cells (Huang & Ives, 1989), this is the first report to demonstrate that the direct intracellular application of GTPγS increased the inward Ca^{2+} channel currents in vascular smooth muscle cells. These studies, therefore, provide valuable information concerning the relationship between G proteins and Ca^{2+} channels.

4.4. Neonatal Rat Ventricular Myocytes

It is known that mammalian cardiac Na^+ channels have a low affinity for TTX ($Kd = 1\ \mu M$) (Cohen *et al.*, 1981) and that these cardiac cells have a large number of Na^+ channels (Bean, 1985). Thus, 10 μM TTX and zero Na^+ were used in the external solution in the present experiments with these cells. No further Na^+ channel current inhibition was achieved by increasing the TTX concentration to 20 μM (Cohen & Lederer, 1988). It is generally believed that in all cardiac cell types, most of the macroscopic Ca^{2+} channel current is carried by L channels (Bean, 1989). However, in the neonatal rat ventricular cell preparation, a T channel component was also observed as described in the results section above.

The results from these experiments clearly showed that GTPγS increased the total inward Ca^{2+} current in neonatal rat ventricular cells. This was consistent with previous reports using other cardiac preparations (Yatani *et al.*, 1987a; Shuba *et al.*, 1990). The effect of GTPγS on Ca^{2+} channel currents was concentration-dependent. It was found that GTPγS not only increased the magnitude of the Ca^{2+} channel currents, but also shifted the peak current of the I-V relationship towards more negative potentials. The increase observed was larger at more negative potentials and smaller at more positive potentials, which was also in agreement with Shuba *et al.* (1990). This is associated with the fact that GTPγS shifted the steady-state activation and inactivation towards more negative potentials. GTPγS increased the L-type and the total (L and T) Ca^{2+} channel current. The effect of GTPγS at a holding potential of -80 mV was greater then that at a holding potential of -40 mV, indicating that GTPγS might also increase the T current in these preparations and this T current may be more sensitive to GTPγS.

In these experiments, the initial current (I_0) was used as a control, which was defined as the time at which the inward current was established, i.e., about 1 minute after the membrane was ruptured. The effect of GTPγS began at 2 minutes after the initial current, which is consistent with other reports. For example, Ito *et al.* (1991) reported that the effect of GTPγS on the outward K^+ channel current reached its peak within 2-4 minutes after rupture of the patch membrane in atrial myocytes. Pollo *et al.* (1991) reported that the inhibitory action of GTPγS on the high threshold Ca^{2+} current in rat sensory neurons was progressive with time. Solutions containing 100 μM GTPγS required 3-4 minutes to reach maximal inhibition in cells with 30 μm in diameter. Complete replacement of the intracellular ionic composition required 5 minutes on average (Doroskenko *et al.*, 1982; Dolphin *et al.*, 1989). Therefore, the effects of GTPγS on inward Ca^{2+} channel currents can be confirmed with the results obtained in experiments using the initial current as a control value (see Method Section 4.1). This was supported by experiments using flash photolysis of caged-GTPγS. The use of caged-GTPγS confirmed the experiments of GTPγS on Ca^{2+} channel currents. The caged-GTPγS was added into the pipette solution. Since it is biologically inactive, the caged-GTPγS would remain inactive after it diffused into the cell and before it was released by photolysis. In the patch clamp study, a reasonable control was, thus, established before using flash photolysis. In addition, the caged-GTPγS can be used to study the onset (time course) of a biological reaction since the photorelease procedures can initiate or terminate a process in milliseconds.

The non-hydrolysable GTP analogue may activate a number of G proteins, including G_s, G_i, G_o and G_p. However, there is considerable evidence suggesting that G_s might be responsible for the increase in Ca^{2+} channel currents. It was reported that cAMP also increased Ca^{2+} channel currents in neonatal rat ventricular cells (Wang *et al.*, 1991b), and G_s is involved in this pathway (Gilman, 1984). Activation of G_s results in the activation of adenylate cyclase, which, in turn, elevates cAMP levels and leads to activation of protein kinase A which phosphorylates Ca^{2+} channel proteins. If this is the case, G_i is not the candidate, because G_i inhibits the adenylate cyclase (Gilman, 1984). It also has been demonstrated that purified G_s or α_s activated cardiac Ca^{2+} channels

(Yatani *et al.*, 1987a). Although G_o was also found in neonatal rat ventricular cells (Silbert *et al.*, 1990), its function is still unknown. G_p has not been reported in neonatal rat ventricular cells. However, protein kinase C can also increase Ca^{2+} channel currents by phosphorylation of the Ca^{2+} channel proteins (Dosenmeci *et al.*, 1988). Therefore, the effect of GTPγS on Ca^{2+} channel currents might be a combination of the activation of several G proteins. Nevertheless, which G protein is responsible for the activation of Ca^{2+} channel currents needs further investigation.

5. Summary

In summary, four types of cells were selected to investigate the effect of GTPγS, a G protein activator, on Ca^{2+} channel currents under the same experimental conditions. The data clearly demonstrate that direct intracellular application of GTPγS increased or decreased VDCC in different tissues, suggesting that G proteins are present in these cells and are involved in the modulation of Ca^{2+} channel currents. These results also indicate that G proteins have different effects on various tissues. The reason for this may be due to the fact that different G proteins are present in the cell membrane. For example, in GH_3 cells, a PTX-sensitive G protein is responsible for the increase in Ca^{2+} channel currents and a CTX-sensitive G protein is responsible for the subsequent decrease of Ca^{2+} channel currents. Different G proteins could also couple to distinct channel proteins. The stimulation of G proteins by GTPγS could, therefore, have different effects on Ca^{2+} channel currents. For example, GTPγS had no effect on T-type channel current in N1E-115 cells, but increased T-type channel current in neonatal ventricular myocytes.

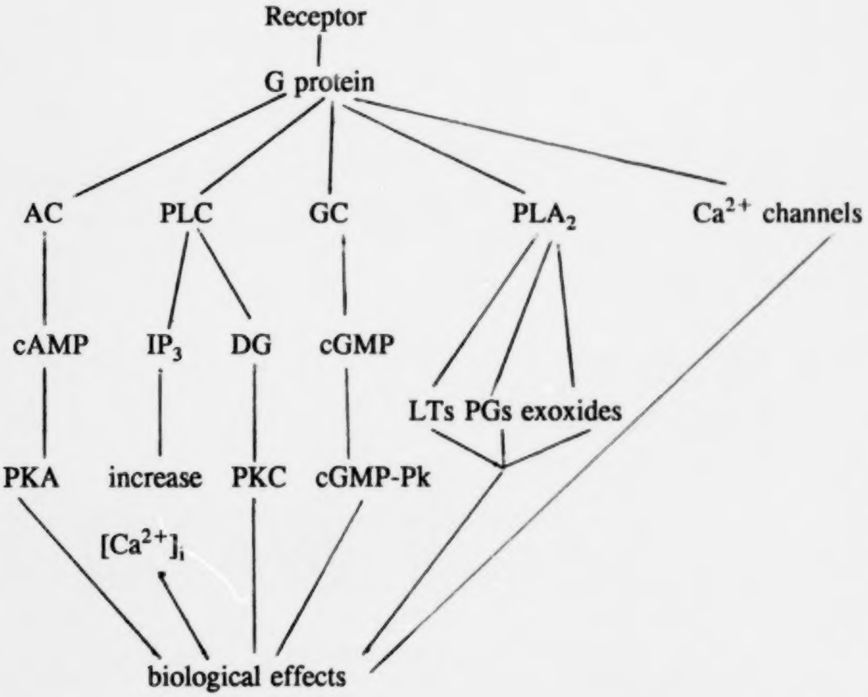
The data in this chapter support the hypothesis that G proteins modulate voltage-dependent Ca^{2+} channels.

CHAPTER IV. α_1-ADRENOCEPTOR ACTIVATION RESULTS IN THE INCREASE OF L-TYPE CALCIUM CHANNEL CURRENT VIA A PTX- AND CTX-INSENSITIVE G PROTEIN AND PKC PATHWAY

1. Introduction

It has been demonstrated (see Chapter III) that G proteins are involved in the modulation of the voltage-dependent Ca^{2+} channels (VDCC). GTPγS, a non-specific G protein activator, activates all G proteins. The effect of GTPγS on VDCC is the summation of the effects of all activated G proteins which are coupled to the channels. Under physiological conditions, G proteins serve as transmembrane transducers and couple the receptors to the effectors (Brown, 1991). In order to study a specific G protein which increases Ca^{2+} channel current in neonatal rat ventricular cells, the α_1-adrenergic receptor was selected. α_1-Adrenoceptors were reported to couple to G proteins (Steinberg *et al.*, 1989) and to activate the L-type Ca^{2+} channel current (Liu *et al.*, 1992b). It is now recognized that a large number of receptors are coupled to effectors by G proteins. Hence, circulating hormones can affect cellular activities without entering cells. Most receptors are transmembrane proteins with agonist binding sites facing outside and one of their intracellular loops connecting to G proteins. The G protein is activated when a receptor binds to its specific agonist. The α subunit of G proteins, when dissociated from the $\beta\gamma$ subunits, activates intracellular enzymes, for example, adenylate cyclase (AC).

Thus, the second messenger cascade is activated to produce biological effects. The diagram below covers several well established second messenger pathways which are controlled by G proteins. It has been demonstrated that PKC (Dosemeci *et al.*, 1988; Lacerda *et al.*, 1988) and PKA (Osterrieder *et al.*, 1982; Curtis & Catterall, 1985) phosphorylate Ca^{2+} channel proteins. The phosphorylation of channel proteins results in an increase or decrease in Ca^{2+} current. Therefore, these two protein kinase pathways are investigated in this chapter.


Receptor
G protein
AC
PLC
GC
PLA$_2$
Ca^{2+} channels
cAMP
IP$_3$
DG
cGMP
LTs PGs exoxides
PKA
increase
PKC
cGMP-Pk
[Ca^{2+}]$_i$
biological effects

LTs: Leukotrienes; PGs: Prostaglandins; PLA_2: Phospholipase A_2

2. Experimental Design

These studies were conducted to investigate a specific G protein pathway (G protein couples the receptor to the effector). The α_1-adrenergic agonist phenylephrine was found to increase the L-type Ca^{2+} channel current in neonatal rat ventricular cells using the whole cell version of the patch clamp technique. Experiments on neonatal rat ventricular tension and action potential shape in a dissociated cell were carried out using phenylephrine to illustrate the significance of the α_1-adrenergic responses in the heart under physiological conditions. Experiments were also designed to investigate the effect of phenylephrine on the T channel current and the sensitivity of the phenylephrine-enhanced current to DHPs. Several α- and β-adrenergic agents were used to show that the phenylephrine effect was a pure α_1-adrenergic action.

Several experimental approaches were used to explore G protein involvement. 1) In the presence of a hydrolysis-resistant GTP analogue, GTPγS, phenylephrine evoked a more pronounced increase in Ca^{2+} current. 2) A non-hydrolysable GDP analogue, GDPβS, blocked the phenylephrine induced increase in Ca^{2+} current. 3) Modification of the G proteins by PTX or CTX altered the α_1-adrenergic response (Trautwein & Hescheler, 1990; Breitwieser, 1991).

The activation of G proteins results in a cascade reaction of many intracellular events. Two protein kinases have been demonstrated to modulate VDCC in neonatal rat ventricular cells. cAMP, as a protein kinase A (PKA) activator, increased the L-type

Ca^{2+} channel current in neonatal rat myocytes (Wang *et al.*, 1991b). Protein kinase C has also been shown to activate the L-type Ca^{2+} channel currents in the same preparations (Dosemeci *et al.*, 1988; Lacerda *et al.*, 1988). In order to determine which kinases mediate the effect of phenylephrine on Ca^{2+} channel current, the activators and inhibitors of these two kinases were used in the experiments.

3. Results

3.1. Characterization of the Action of an α_1-Adrenergic Agonist, Phenylephrine, on L-Type Calcium Channel Current

3.1.1. *Positive Inotropic Effects of Phenylephrine on Neonatal Rat Ventricles*

The concentration-dependent positive inotropic effect of phenylephrine is shown in Fig. IV-1. The experiments were performed using neonatal rat ventricles (complete ventricle) which were stimulated with depolarizing pulses at a rate of 4 Hz. The changes in developed tension produced by phenylephrine were expressed as a percentage of the control (obtained in the absence of the drug). Phenylephrine produced a positive inotropic effect in the concentration range of 10^{-7} to 10^{-4} M with an EC_{50} of 3.5 μM, which is consistent with previous reports (Wagner & Reinhardt, 1974, Chess-Williams & Broadley, 1987). In an attempt to explain the positive inotropic effect, current clamp experiments were carried out using single dissociated cells from neonatal rat ventricles. The cells were stimulated using constant current injection, 1.5 times threshold at a rate of 0.1 Hz from a potential of -80 mV. The cells were exposed to 10^{-5} M phenylephrine.

Every 5 minutes, the effect of the drug was recorded. Fig. IV-2A. shows that phenylephrine caused an increase in the plateau phase of the action potential which is similar to that caused by Bay K 8644 (1 μM) (Fig. IV-2B). The K^+ channel blocker, 4-AP (2 mM), also prolonged the plateau phase of the action potential. The change in the plateau after 4-AP was greater than the change after phenylephrine and Bay K 8644. The action potentials after 4-AP were characterized by a very long plateau phase (Fig. IV-2C). These results suggest that phenylephrine might prolong the plateau phase of the action potential by increasing the inward Ca^{2+} current.

3.1.2. *Effect of Phenylephrine on the L-Type Ca^{2+} Channel Current*

Whole-cell voltage-clamp experiments were employed to investigate the effect of the α_1-adrenergic agonist phenylephrine on the L-type Ca^{2+} channel current. Since the L-type Ca^{2+} channel is more permeable to Ba^{2+} than to Ca^{2+} (Tsien, 1983), Ba^{2+} was used as a charge carrier. Experiments (data not shown) indicate that no further sodium channel blockade was achieved by increasing TTX concentration above 20 μM. Therefore, 20 μM TTX was used in the experiments. When the inward current was activated by the application of a depolarizing pulse to various potentials (250 ms duration) from a holding potential of -40 mV, addition of 10 μM phenylephrine to the bath solution increased the inward current. This augmentation of the current was observed at more negative potentials. In addition, it was also observed that the inactivation process after phenylephrine was faster and the tail current kinetics also changed (Fig. IV-3A). The I-V relationship before and after phenylephrine shows that the effect of phenylephrine was more prominent at negative potentials (Fig. IV-3). Furthermore, phenylephrine shifted the maximal amplitude of the current in the I-V relationship by about 10 mV towards more negative potentials. In order to clearly demonstrate the effect of phenylephrine on the Ca^{2+} channel current at the different test potentials, the inward currents before and after phenylephrine were measured and compared and then plotted against test voltages (Fig. IV-3C). The results showed that phenylephrine significantly increased the L-type Ca^{2+} channel current at more negative potentials. The greatest augmentation was between -20 to +20 mV. This was repeated in five cells. Because the current values at -20 mV were very small in the absence of phenylephrine, the augmentation of the current by phenylephrine at these voltages was much greater.

Fig. IV-4 shows the concentration-dependence of the action of phenylephrine on the L-type current. The current was evoked by a depolarizing pulse to 0 mV from the holding potential of -40 mV. In the presence and absence of phenylephrine, the current was measured and compared using the same test pulse magnitude. The increase was converted to a percentage of maximum increase and plotted against the phenylephrine concentrations. The curve was fitted to the Hill equation and plotted as percentage of maximal increase versus phenylephrine concentration. Phenylephrine increased the L-type Ca^{2+} channel current in a concentration-dependent manner. The concentration required to produce the half maximal response (ED_{50}) was $6x10^{-7}$ M.

The augmentation of the L-type Ca^{2+} channel current by phenylephrine occurred slowly under the conditions of this study. The response started at about 7 minutes after administration of phenylephrine into the bath solution. The time varied according to the concentrations applied. The time for maximal response was about 10-15 minutes after the application of phenylephrine. Furthermore, it was not possible to abolish the phenylephrine-induced increase in inward Ca^{2+} current by washing out the bath solution for 10 minutes at a rate of 0.5 ml/minute. However, 10 μM nifedipine completely blocked this inward current as described below (Fig. IV-5).

3.1.3. *Effect of Nifedipine on the L-Type Ca^{2+} Channel Current Increase Induced by Phenylephrine*

When the holding potential was -40 mV, most of the T-type current was inactivated and only the L-type current remained. This L-type current was sensitive to the dihydropyridine, nifedipine. Therefore, nifedipine was used in order to determine whether or not the total L-type Ca^{2+} channel current was sensitive to the dihydropyridine. The total current is the current activated by depolarization plus the additional current induced by phenylephrine. In cells pretreated with 10 μM phenylephrine for 10 minutes (n=5), 10 μM nifedipine completely blocked the total L-type Ca^{2+} channel current (Fig. IV-6). Therefore, phenylephrine may open more DHP-sensitive L-type Ca^{2+} channels and/or make them open more frequently, which is in agreement with a previous report (Rand *et al.*, 1986).

3.1.4. *Effect of Phenylephrine on the Steady-State Inactivation and Activation of the L-Type Ca^{2+} Channel Current*

In order to determine if the phenylephrine effect was membrane potential dependent, complete steady-state inactivation curves were generated. The steady-state inactivation of the L-type channel current was examined before and after addition of 10^{-5} M phenylephrine. The voltage clamp protocol is shown in the inset of Fig. IV-7. The membrane was clamped at various conditioning potentials for 1 second. This was followed by a test pulse to +10 mV for 200 ms. The amplitudes of the inward current evoked by the test pulses were normalized to the evoked current at the most negative conditioning potential. In both cases, control and phenylephrine adminstration, the currents were reduced in amplitude at a conditioning potential of -30 mV. The channels were usually completely inactivated at about +10 mV (phenylephrine) or +5 mV (control). The Boltzmann function of the form $I=I_{max}[1+\exp(V-V_h)/k]^{-1}$ was used to fit the experimental data shown in Fig. IV-7. In this equation, V represents the holding potential, V_h is the potential at which one half of the calcium channels are inactivated and k is the slope factor. Phenylephrine increased current at all conditioning potentials and shifted the steady-state inactivation curve to the left by about 4 mV. V_h(control)=-7.3 mV, V_h(phenylephrine)=-11.4 mV. The slopes of the two curves as determined from the curve fit were similar ($k_1=5.9$; $k_2=6$). Almost identical results were obtained from three other cells. Similar results have been described for the dihydropyridine agonist Bay K 8644 (Wang *et al.*, 1989).

The instantaneous I-V curve for the L-type channel in neonatal myocytes is not linear. Hence, the steady-state activation cannot be accurately represented as a conductance (Hagiwara & Byerly, 1981). In order to estimate the phenylephrine-induced shift in the voltage dependence of L channel activation, tail currents were used. A characteristic of the L-type inward current with Ba^{2+} as the charge carrier in these cells is a slow decline during a depolarizing test pulse. Fig. IV-8A shows the slow decline in the L-type current evoked by a 250 ms test pulse to +10 mV and the superimposed tail currents of five pulses (50 ms increments) of increasing duration. The magnitude of the tail currents and the envelope decay with similar time courses suggesting that the tail current predominantly represents the inward movement of Ba^{2+} through Ca^{2+} channels. In order to estimate the steady-state activation of the L-type channel current, the membrane potential was held at -40 mV and a test pulse to various potentials was applied. The tail current was measured when the membrane potential returned to the holding potential (-40 mV) as shown in Fig. IV-8B. The tail currents at all test potentials were normalized to the maximal tail current. The normalized tail currents were plotted against various test potentials and these results before and after phenylephrine administration are shown in Fig. IV-8C. Phenylephrine shifted the activation of the L-type Ca^{2+} channel current in this cell by 10 mV towards more negative potentials. Similar results were obtained in four other cells.

3.1.5. *Effect of Phenylephrine on the L-Type Ca^{2+} Channel Current Via the α_1-Adrenergic Receptor*

In order to demonstrate that the effect of phenylephrine on the L-type current is modulated by α_1-adrenergic receptors, several adrenergic agonists or antagonists were used.

Prazosin, an α_1-adrenergic antagonist, was used to eliminate the effect of phenylephrine on the L-type current. Fig. IV-9A shows that after addition of the same concentration of prazosin prior to phenylephrine, the increase in inward current was abolished. Since prazosin and phenylephrine bind to the same receptor and this binding is rapid, prazosin was added into the bath immediately before the addition of phenylephrine. The results clearly show that prazosin abolished the augmentation of the inward L-type current induced by phenylephrine, indicating that the effect of phenylephrine is through the α_1-adrenergic receptor. Prazosin by itself was found to have no effect on the L-type current (data not shown).

Further experiments were carried out to determine whether the effect of phenylephrine on the L-type current is exclusively modulated by the α_1-receptor. Clonidine, an α_2-adrenergic agonist, was used to investigate the α_2-adrenoceptor effect on the L-type current. Fig. IV-9B shows that 10 μM clonidine did not increase or decrease the L-type current in neonatal rat ventricular cells. This experiment was repeated three times. Yohimbine, an α_2-adrenergic antagonist, was added to the bath solution prior to phenylephrine to determine the effect of the α_2-adrenergic antagonist. These experiments ($n=3$) demonstrated that yohimbine did not abolish the effect of phenylephrine on the L-type current (Fig. IV-9C). Furthermore, it has been reported that α_2-adrenoceptors are not present in ventricular cells (Buxton & Brunton, 1986). Therefore, the conclusion from these studies was that the effect of phenylephrine on the L-type current was mediated by α_1-adrenergic receptors.

It is known that phenylephrine is not a completely specific agent for the α_1-adrenoceptors and to exclude the possibility that the effect of phenylephrine was via the

β-adrenergic receptor, the β-antagonist propranolol was used. The results from four cells showed that propranolol did not abolish the effect of phenylephrine on the L-type current, indicating that the effect of phenylephrine on the inward Ba^{2+} current was not via the β-receptor (Fig. IV-9D). In addition, it has been reported that the α_1-receptor was predominant in neonatal rat ventricular cells (Chess-Williams *et al.*, 1990) and thus phenylephrine must exert its effect on the L-type current through the α_1-adrenergic receptor. Propranolol by itself had no direct effect on the L-type current (data not shown).

3.1.6. *Effect of Phenylephrine on the T-Type Ca^{2+} Channel Current*

Both T- and L-type Ca^{2+} channels have been identified previously in neonatal rat ventricular cells (Wang *et al.*, 1991a; Liu *et al.*, 1992b). In the present experiments, depolarizing the cells to a test potential of 0 mV from a holding potential of -80 mV evoked both T- and L-types of currents (Fig. IV-10A). Nifedipine blocked the L-type Ca^{2+} channel current, as has been reported previously (Brown *et al.*, 1986) and did not affect the T-type Ca^{2+} channel current. Fig. IV-10 shows that 1 μM nifedipine blocked most of the L-type current. In cells pretreated with nifedipine (1 μM), 10 μM phenylephrine increased the Ca^{2+} current. However, addition of another 3 μM nifedipine brought the current back to the original level (control level). This effect of phenylephrine might be due to the fact that the concentration of nifedipine (1 μM) was not enough to block the L-type current completely. Therefore, some L-type Ca^{2+} channel current remained and phenylephrine may act on the remaining part of the L-type channel. Further experiments using a higher concentration of nifedipine (10 μM) were conducted to eliminate the possibility that phenylephrine modulated the T-channel current. Fig. IV-11 shows that after addition of 10 μM nifedipine, phenylephrine did not affect the T-type current. Therefore, these experiments clearly demonstrate that the stimulation of the α_1-adrenoceptors by phenylephrine resulted in the increase in L-type Ca^{2+} channel currents in neonatal rat ventricular myocytes as shown in the diagram (Fig. IV-11).

Figure IV-1.

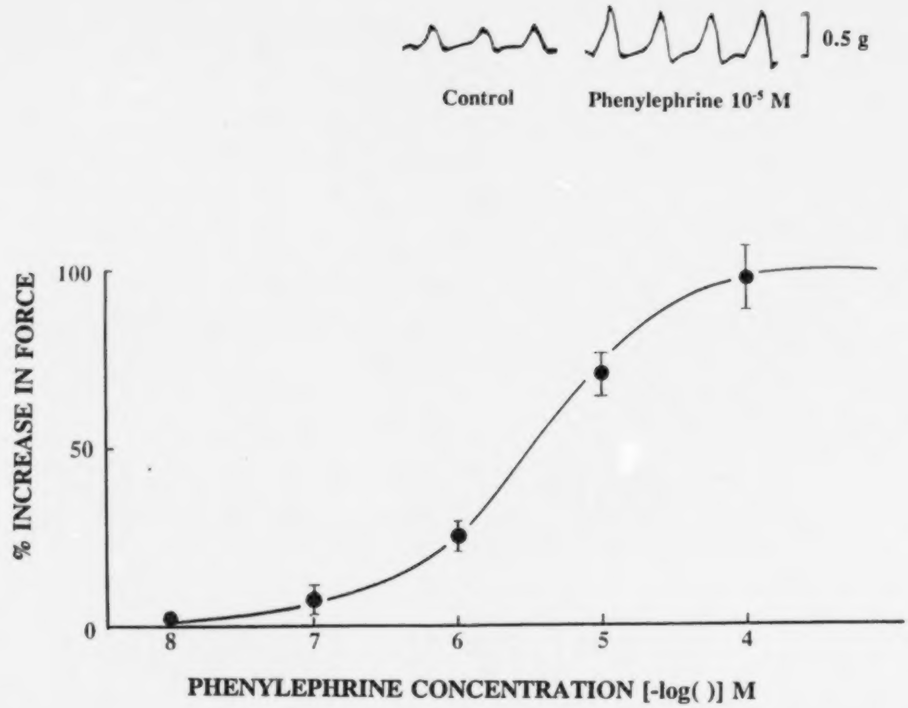


Fig. IV-1. The concentration-dependent inotropic response induced by phenylephrine in neonatal rat ventricle. The ventricles were driven electrically at a rate of 4 Hz. The inset shows the original tension recordings. 10 μM phenylephrine significantly increased the tension. The increase in tension recorded after different concentrations of phenylephrine were expressed as a percentage of the control values (mean±S.E.M.), which were reported as % of maximal increase. The number of cells tested at each concentration ranged from 3-4. The curve was fitted using the Hill equation as described in the Methods, Section (7).

Fig. IV-2.

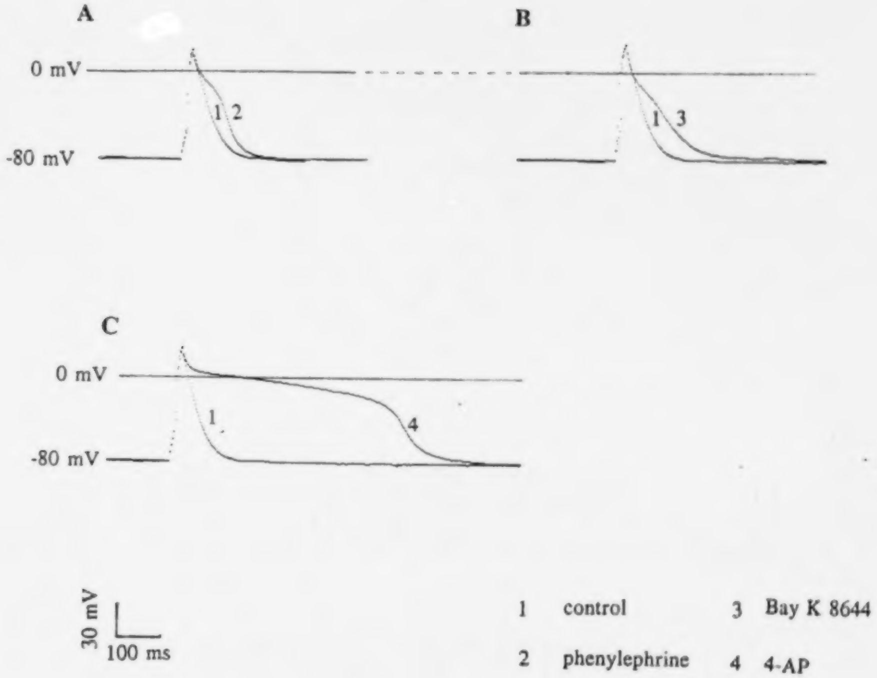


Fig. IV-2. Effect of phenylephrine on the neonatal rat ventricular action potential. Action potentials were evoked at 10-sec intervals by 15 ms superthreshold depolarizing current pulses delivered through the recording pipette. A. 10^{-5} M phenylephrine prolonged the plateau phase of the action potential. B. Similar results were obtained using 1 μM Bay K 8644. C. The K^+ channel blocker, 4-AP, prolonged the plateau phase of the action potential but the pattern was different. 1) is the control action potential, 2) is the action potential after phenylephrine, 3) is the action potential after Bay K 8644, and 4) is the action potential after 4-AP.

Figure IV-3.

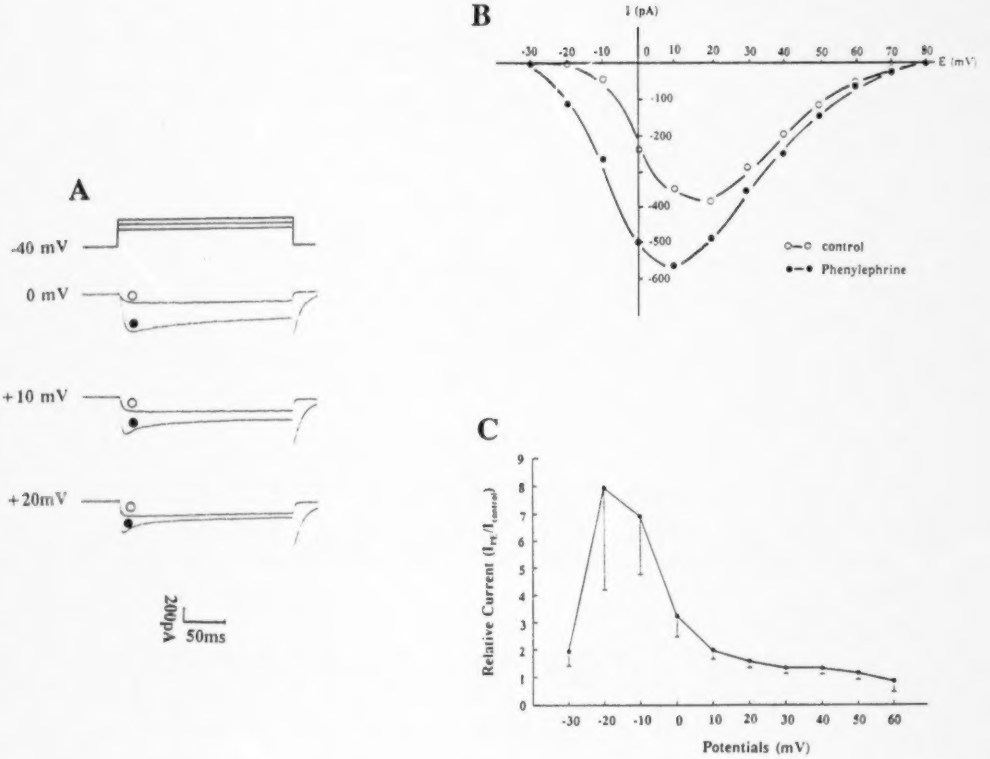


Fig. IV-3. Effect of 10 μM phenylephrine on the inward L-type current. The myocytes were held at -40 mV to inactivate most of the T current. A. Current records from a holding potential of -40 to three different test potentials. Filled circles represent 10 μM phenylephrine and open circles represent control records. Phenylephrine significantly increased the L-type current. Note that the inactivation was more rapid and the tail current was altered. B. The current voltage relationship was plotted from the same cell shown in A. Note that the enhancement of the inward L-type current occurred at the more negative potentials. C. Phenylephrine increased the L-type current more in the negative potential range. Values are expressed as mean±S.E.M. (n=5).

Figure IV-4.

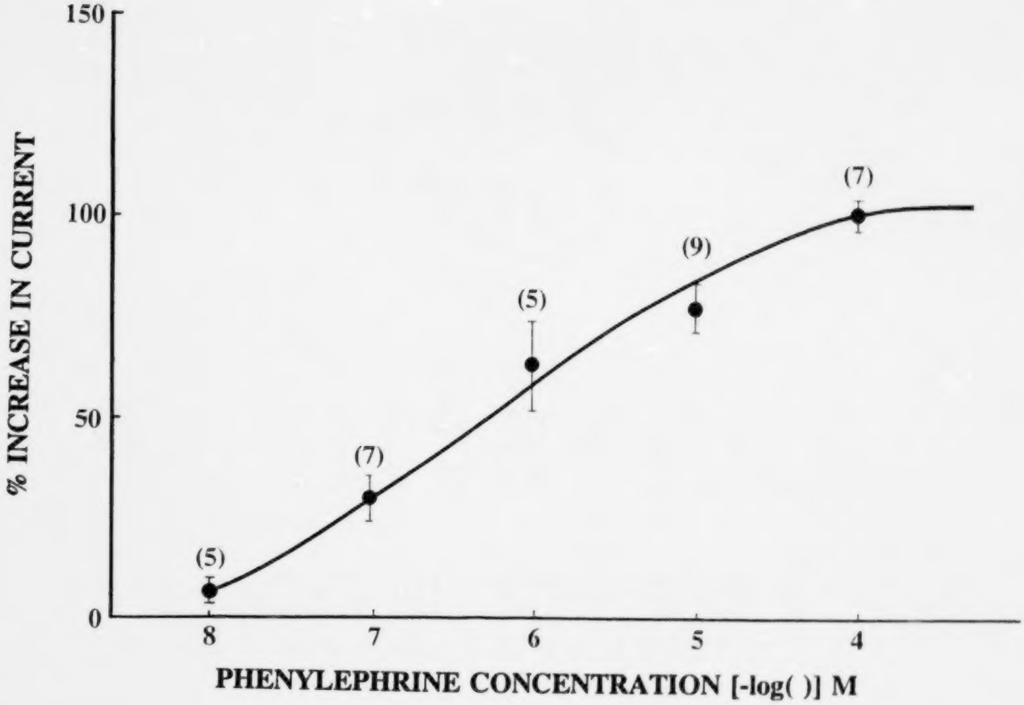


Fig. IV-4. Effect of different concentrations of phenylephrine on the magnitude of the inward L-type current. Voltage clamp pulses were applied from -40 to 0 mV (250 ms duration) at 0.1 Hz. Current amplitudes were obtained after the cell was exposed to the different concentrations of the drug. Each concentration was only tested on one cell. Current is expressed as the percentage of control, which was reported as % of maximal increase. The number adjacent to each point indicates the number of cells studied. Values are expressed as mean ± S.E.M. The curve was fitted using the Hill equation as described in the Methods, Section (7).

Figure IV-5.

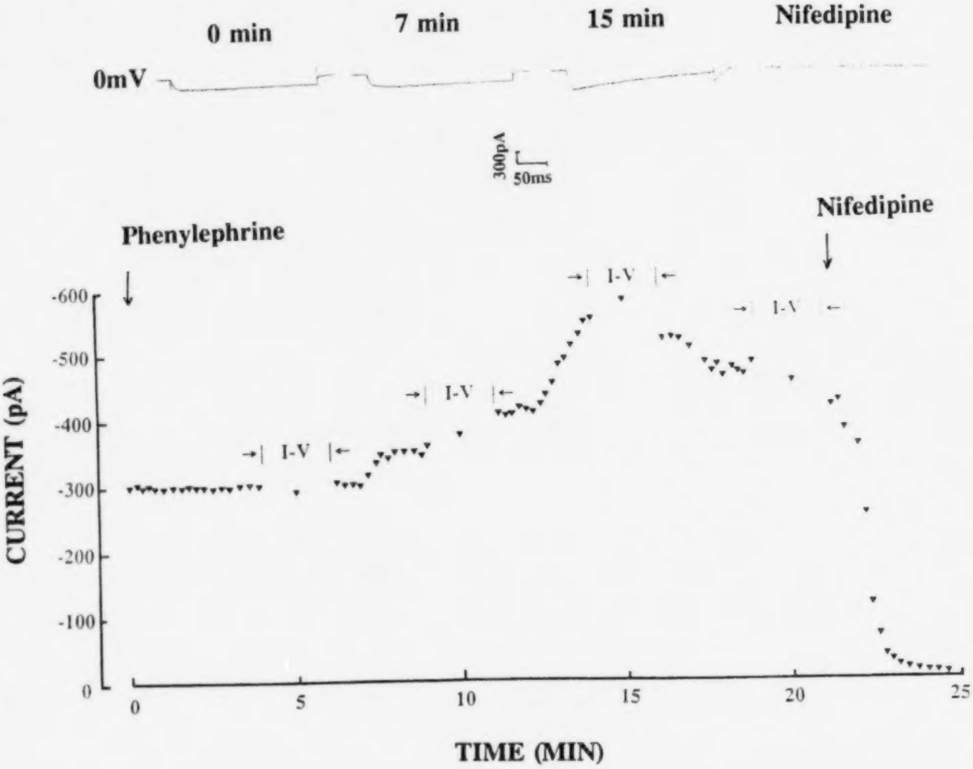


Fig. IV-5. The time-dependent effect of phenylephrine on the L-type current. 10 μM phenylephrine was added into the bath solution after the current reached the steady-state. The upper traces are original recordings (leakage corrected). Phenylephrine increased the L-type current beginning at 7 min and reached the peak at about 15 min.

Fig. IV-6.

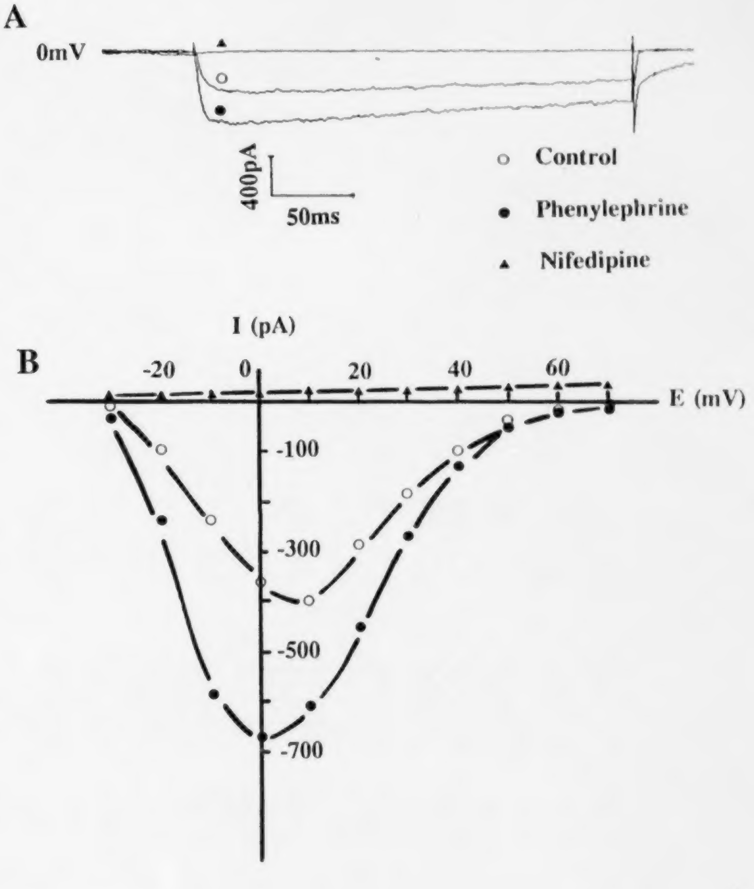


Fig. IV-6. Effect of nifedipine on the phenylephrine-enhanced inward L-type current. A: original current records (leakage subtracted). B: the current voltage relationship before and after addition of phenylephrine (10 μM) and nifedipine (10 μM), plotted from the same current records shown above.

Figure IV-7.

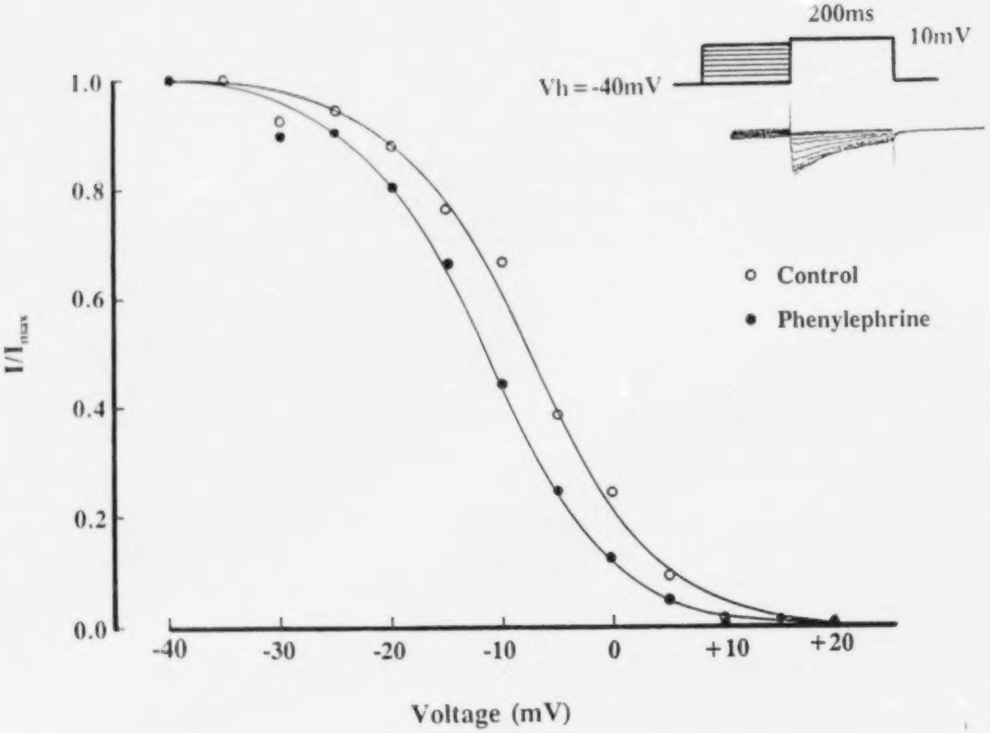


Fig. IV-7. Effect of phenylephrine on the steady-state inactivation of the L-type current. The protocol is shown in the inset on the top right corner. A 1 sec conditioning pulse was followed by a 200 ms test pulse. Phenylephrine shifted the steady-state inactivation curve towards more negative potentials. The curve was fitted by the Boltzmann equation as described in the Methods section (7).

Figure IV-8.

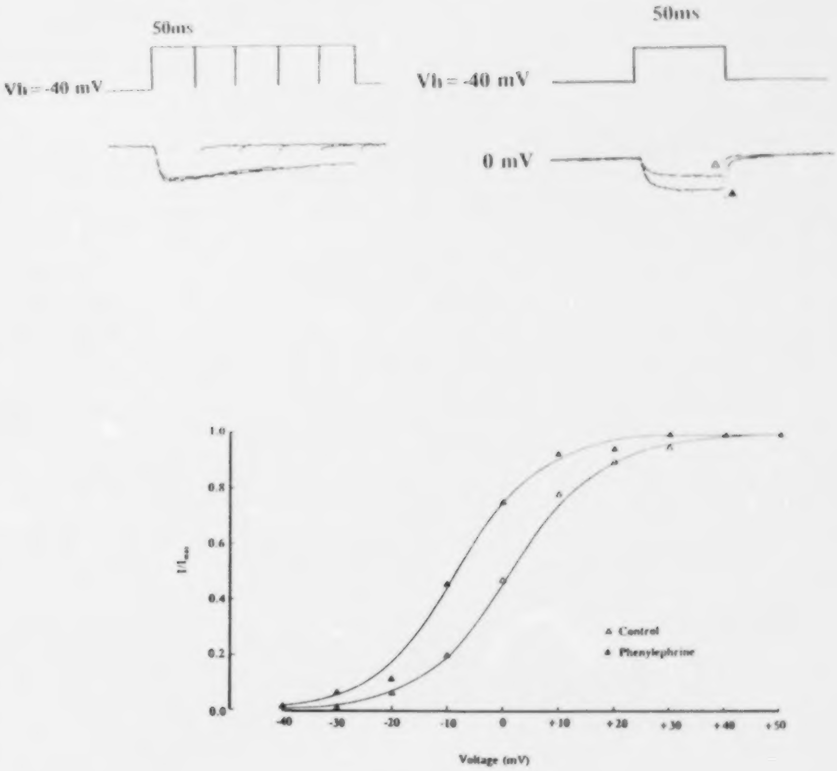


Fig. IV-8. Effect of phenylephrine on the steady-state activation of the L-type Ca^{2+} channel current. A. Tail current envelope recorded from a holding potential of -40 mV to a test pulse of +10 mV with a progressively increasing duration of 50 ms. B. Protocol for measuring the tail current in the presence (filled triangles) and absence of phenylephrine (opened triangle). C. Normalized steady-state activation curve in the presence and absence of phenylephrine. Phenylephrine shifted the curve towards negative potentials. The curve was fitted by the Boltzmann equation as described in the Methods section (7).

Figure IV-9.

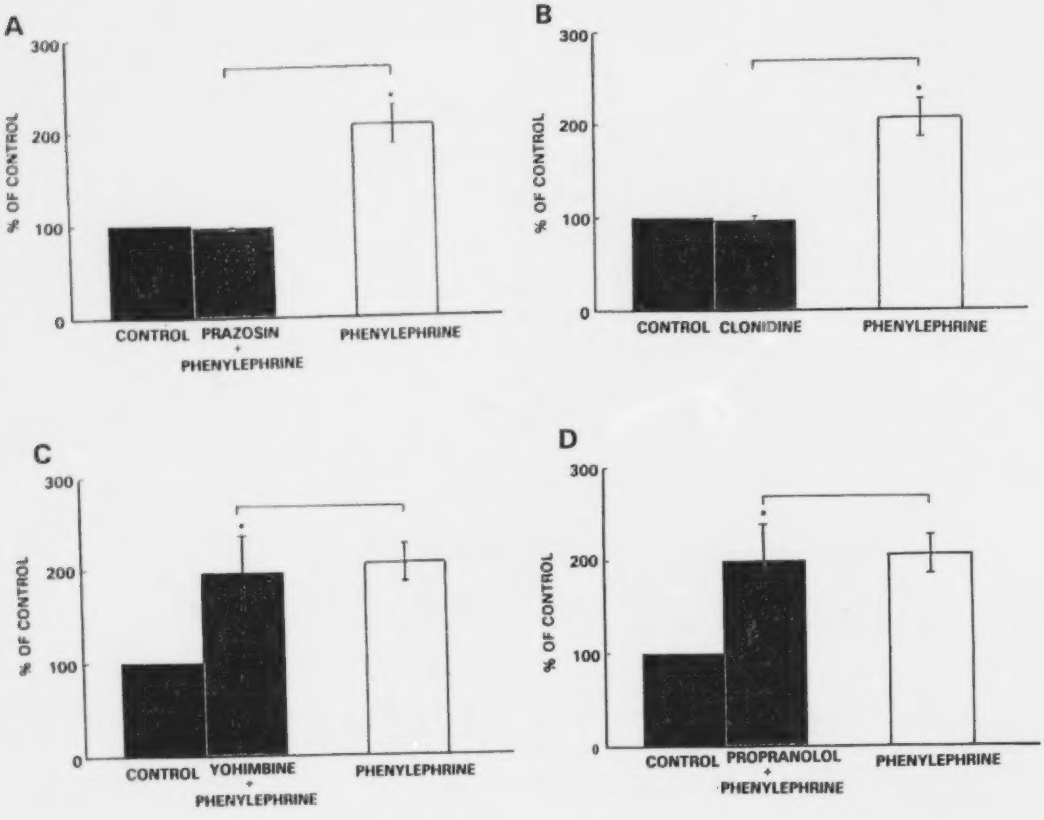


Fig. IV-9. A: Effect of adrenergic agonists and antagonists on the L-type current and comparison of these effects with an equimolar concentration of phenylephrine. A: Prazosin (10^{-5} M) abolished the effect of phenylephrine on the L-type current in neonatal rat ventricular cells ($n=4$). Prazosin was applied prior to phenylephrine. B: The α_2-agonist clonidine did not increase or decrease the L-type current in neonatal rat ventricular cells ($n=3$). C: The α_2-antagonist yohimbine did not abolish the phenylephrine-enhanced Ca^{2+} channel current ($n=3$). Yohimbine was applied prior to phenylephrine. D: Effect of the β-antagonist propranolol on the phenylephrine-enhanced Ca^{2+} channel current. Propranolol was applied prior to phenylephrine. Phenylephrine increased the L-type current in the presence of propranolol (10^{-5} M) ($n=4$). In all four figures, $*P<0.05$. The values are expressed as mean±S.E.M.

Figure IV-10.

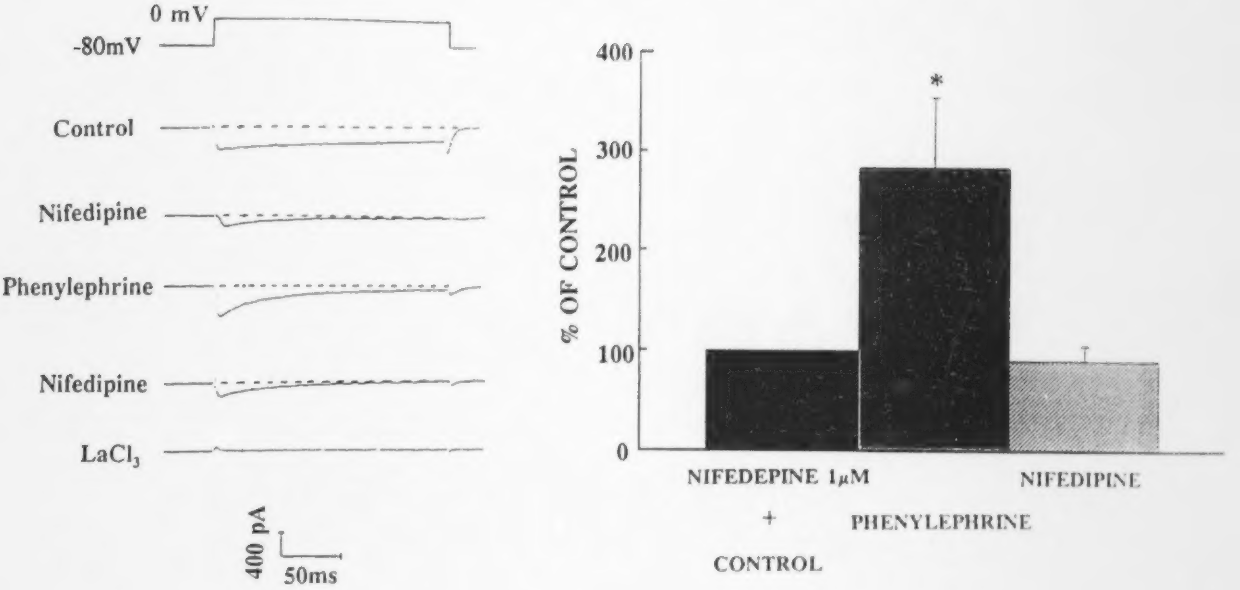


Fig. IV-10. After 1 μM nifedipine blocked the L-type current, 10 μM phenylephrine increased the Ca^{2+} channel current in the presence of 1 μM nifedipine. However, this phenylephrine-induced enhancement of the L-type current can be further blocked by addition of 3 μM nifedipine. Values are expressed as mean±S.E.M (n=4)

Figure IV-11.

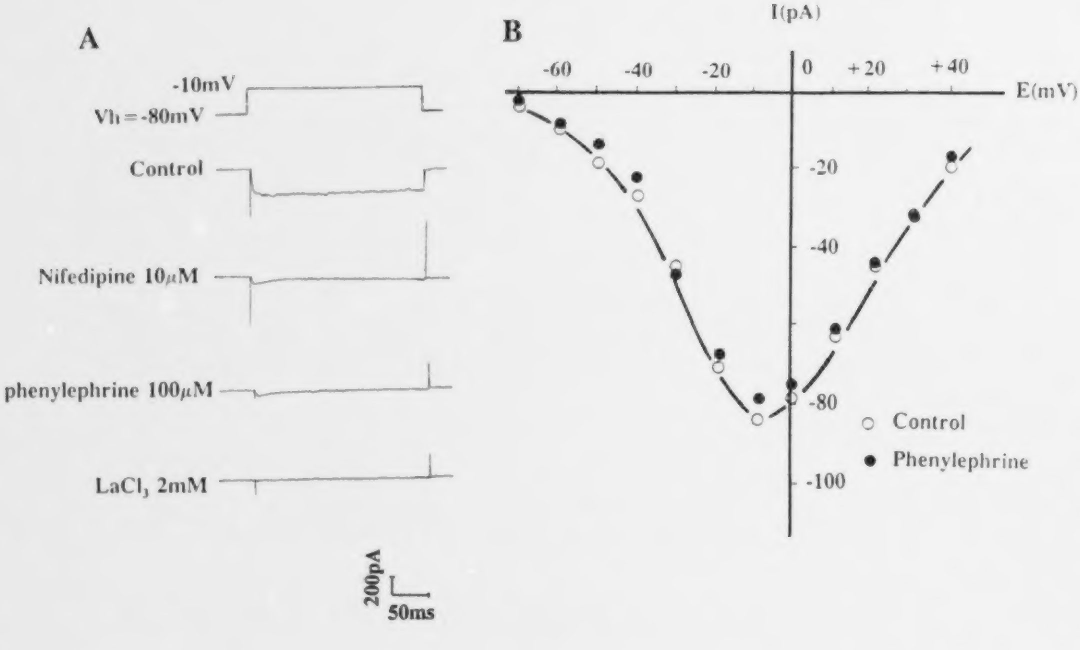


Fig. IV-11. Phenylephrine did not modify the T-type current. 10 μM nifedipine was applied to block the L-type current. In the presence of 10 μM nifedipine, phenylephrine was added to the bath solution. The T-type current was not affected by phenylephrine during the 20 min observation period. La^{3+} (2 mM) completely blocked the T-Type Ca^{2+} current.

Figure IV-12.

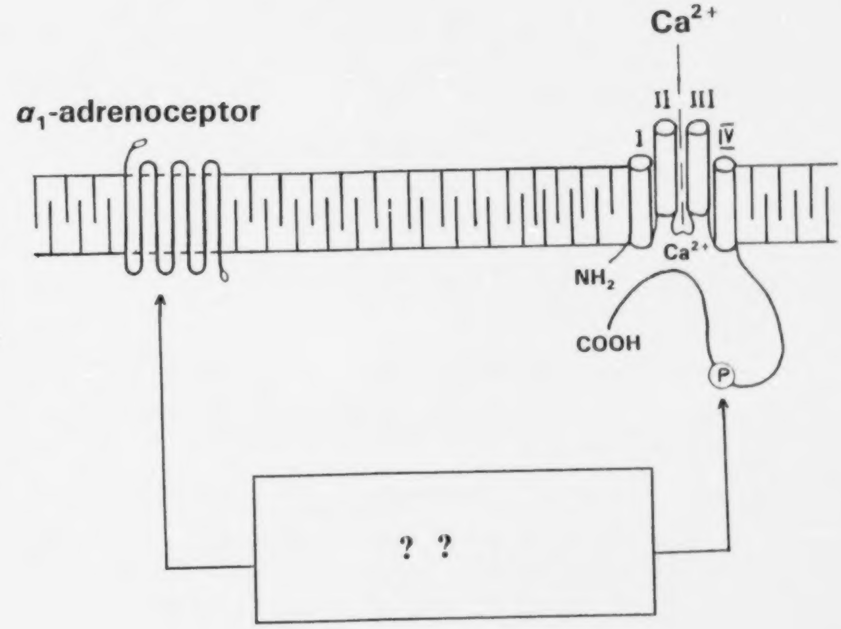

α_1-adrenoceptor
Ca^{2+}
I
II
III
IV
Ca^{2+}
NH_2
COOH
P
? ?

Fig. IV-12. Schematic diagram showing that stimulation of the α_1-adrenoceptor in neonatal rat ventricular cells increases L-type Ca^{2+} channel current, as demonstrated in the results by an undetermined mechanism.

3.2. Role of G Protein in the Phenylephrine-Enhanced L-Type Calcium Channel Current in Neonatal Rat Ventricular Cells

3.2.1. *The Potentiation Effect of GTPγS on Phenylephrine-Enhanced L-Type Current*

One means by which coupling of G proteins to their receptors can be assessed is the determination of the ability of guanine nucleotides, such as GTPγS, to increase the increase in Ca^{2+} current. The effect of the intracellular application of GTPγS on VDCC currents occurred 2-3 minutes after the initial current (control current) was established as shown in Chapter III. Therefore, in the following experiments, phenylephrine was usually added to the bath 10 minutes after rupture of the membrane. Hence, there would be sufficient time for the pipette solution to completely exchange with the cytosol and for GTPγS to activate the G proteins. The cells were discarded if the membrane inward current did not reach steady-state within 10 minutes. The effect of intracellular application of GTPγS on the phenylephrine-enhanced Ca^{2+} channel current is shown in Fig. IV-13. The control current was obtained when intracellular dialysis was complete (10 minutes). At the end of this equilibration period, phenylephrine was added to the bath. Ten minutes after addition of the drug, the currents were recorded and normalized to the control current. The concentration-dependent curve was shifted to the right in the presence of GTPγS, suggesting that GTPγS enhanced the phenylephrine effect. This enhancement was concentration dependent (10^{-8}-10^{-6} M). The values are expressed as a percentage of increase. In chapter III, GTPγS was also observed to increase Ca^{2+} channel current in neonatal rat ventricular cells. This effect of GTPγS on Ca^{2+} channel current was similar to that of phenylephrine. Therefore, these experiments suggest that G proteins might be involved in the phenylephrine-induced increase in Ca^{2+} channel current.

3.2.2. *Blocking Effect of GDPβS on the Phenylephrine-Induced Increase in Ca^{2+} Current*

A non-hydrolysable analog of GDP, GDPβS, can be used to block the G protein mediated effects of neurotransmitters or hormones on Ca^{2+} channel currents (Holz *et al.*, 1986; Dolphin & Scott, 1987). Thus, the effect of GDPβS on the phenylephrine-enhanced L-type current was studied. The method used was similar to that described for GTPγS. After rupture of the cell membrane, a period of 10 minutes was allowed to insure adequate internal dialysis with GDPβS. The results show that inclusion of 1 mM GDPβS in the pipette completely blocked the phenylephrine-induced increase in L-type Ca^{2+} channel current (Fig. IV-14). The upper panel in Fig. IV-14 shows the original current records from two cells which were activated by depolarizing the cell to 0 mV from a holding potential of -40 mV. One cell was dialysed with GDPβS. The other cell was dialysed with normal pipette solution. The effect of phenylephrine (100 μM) on L-type current was totally abolished in the cell dialysed with GDPβS. The histogram (bottom) shows that the phenylephrine (100 μM) -induced L-type current increase was completely blocked by the inclusion of GDPβS in the pipette solution. The current amplitude after phenylephrine was 104.1±8.3% (n=4) in the cells dialysed with GDPβS. This was significantly different from the normalized current in the cells dialysed with normal solution which was 216.9±7.1%. Therefore, the data from these two experiments, GTPγS enhancement and GDPβS inhibition of the L-type Ca^{2+} current induced by phenylephrine, confirmed the involvement of a G protein in the modulation of Ca^{2+} channel current.

3.2.3. *Effect of CTX Pretreatment on the Phenylephrine-Induced Increase in Ca^{2+} Current*

CTX persistently activates G proteins by causing the covalent transfer of ADP-ribose from NAD to the α subunit of G_s (Murakami & Yasuda, 1986). CTX maximally activates G_s which, in turn, completely activates adenylate cyclase. This will mask the effect of a stimulatory hormone. If a substrate for CTX links the α_1-adrenoceptor to the L channel protein, it would be expected that the phenylephrine effect would be masked since the CTX-sensitive G protein is maximally activated. Therefore, the effect of CTX was studied by pretreatment of cells with CTX for 12 hours. The effect of CTX (1 μg/ml) pretreatment on the L-type Ca^{2+} channel current is shown in Fig. IV-15. The original current records from two cells which were activated by depolarizing the cell to 0 mV from a holding potential of -40 mV are shown at the top of the figure. One cell was pretreated with CTX and the other cell was not. The phenylephrine enhanced L-type current was not abolished by CTX. Studies using a group of cells also confirmed this effect as shown in Fig. IV-15 (bottom). The data was analyzed as described above. Phenylephrine increased the L-type current significantly in the cells pretreated with CTX. The normalized current induced by phenylephrine after CTX pretreatment was $216.9 \pm 38.1\%$ ($n=5$) which was not significantly different from the normalized current in cells not pretreated with CTX ($216.7 \pm 7.1\%$, $P>0.05$). Therefore, the effect of phenylephrine on the L-type Ca^{2+} channel current is not mediated by a CTX-sensitive

G protein. The results from these experiments suggest that the G protein involved in this effect is a CTX-insensitive G protein.

3.2.4. *The Effect of PTX Pretreatment on the Phenylephrine-Induced Increase in Ca^{2+} Current*

PTX inhibits the function of many G proteins causing the covalent transfer of ADP-ribose from NAD to the α subunit of G_i or G_o (Moss, 1987). If a substrate for PTX links the α_1-adrenoceptor to the L-type channel protein, it would be expected that the response to phenylephrine would be abolished by PTX pretreatment. The effect of phenylephrine on the L current in neonatal ventricular cells pretreated with PTX (200 ng/ml) is shown in Fig. IV-16. The current records from two cells, one with and the other without PTX pretreatment are shown in Fig. IV-16 (top). The currents were elicited from a holding potential of -40 mV to a test potential of 0 mV. The phenylephrine induced increase in the L-type current was obtained in the presence of PTX pretreatment. Phenylephrine significantly increased the normalized L-type current to $214.6 \pm 30.1\%$. This is similar to the increase induced by phenylephrine which was measured in a group of cells not pretreated with PTX ($216.7 \pm 7.1\%$). Therefore, the effect of phenylephrine on L-type Ca^{2+} current was not mediated by a PTX-sensitive G protein. The results from these experiments suggest that the G protein coupling α_1-adrenoceptor and the L-type Ca^{2+} channel is a PTX-insensitive G protein. This is in agreement with the report of Han *et al.* (1989).

The results obtained above indicate that the G proteins are the components that couple the α_1-adrenoceptors and the L-type Ca^{2+} channels. When the stimulation of the

α_1-adrenoceptor occurs, the chain of signal-transferring steps proceeds and causes the modulation of Ca^{2+} channel activity. Fig. IV-17. shows a schematic diagram of this three-element mechanisms (Rosenthal & Schultz, 1987).

Figure IV-13.

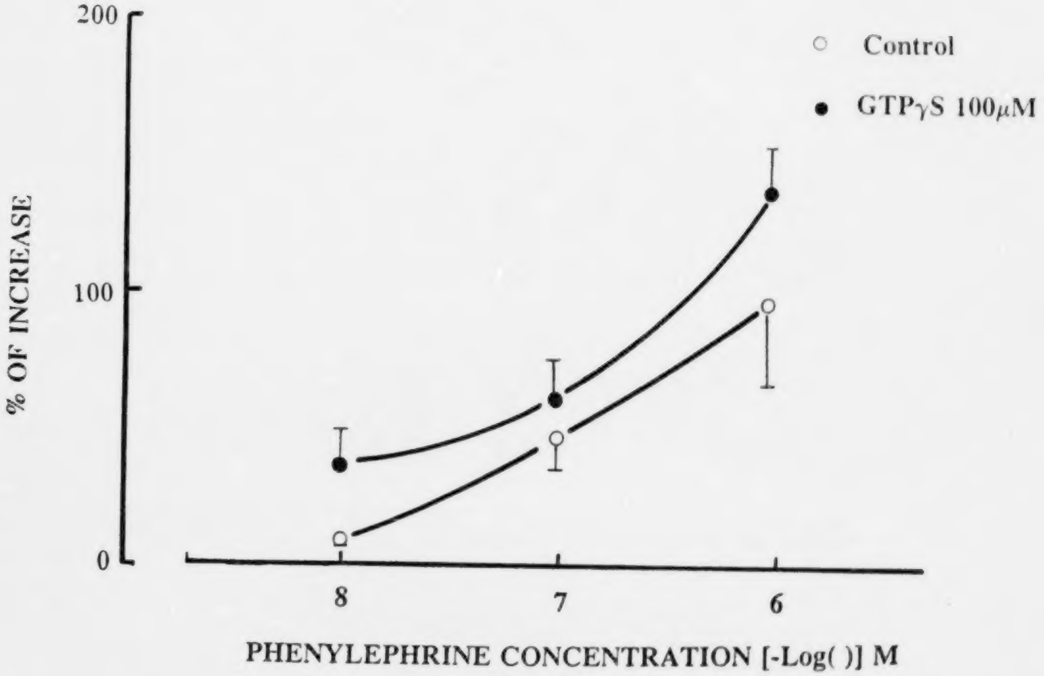


Fig. IV-13. GTPγS enhances the phenylephrine-induced increase in L-type current. Values are expressed as percentage of the increase and plotted against phenylephrine concentration (mean±S.E.M). Open circles represent a control group of cells and filled circles represent the cells which were dialysed with 100 μM GTPγS. The numbers next to the points are the numbers of cells tested.

Figure IV-14.

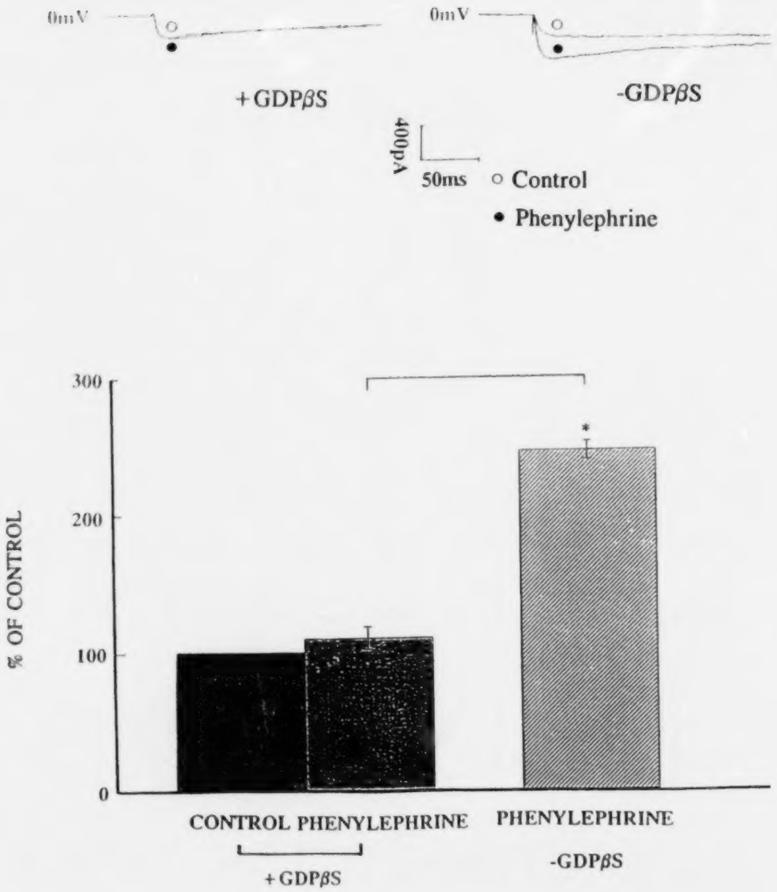


Fig. IV-14. GDPβS completely blocked the phenylephrine-induced increase in L-type current. Upper panel shows original current records from two cells which were activated by depolarizing the cell from a holding potential of -40 mV to a test potential of 0 mV. In the presence of 1 mM GDPβS in the pipette solution, the effect of phenylephrine (10^{-4} M) was totally abolished. Without GDPβS in the pipette solution, phenylephrine increased the Ca^{2+} channel current. Studies using groups of cells confirmed this effect shown at the bottom of the figure. The current increase from the cells (n=7) without GDPβS in the pipette is significantly different from that with GDPβS in the pipette (n=4). Values are mean±S.E.M. *$P<0.05$ (Student's t test).

Figure IV-15.

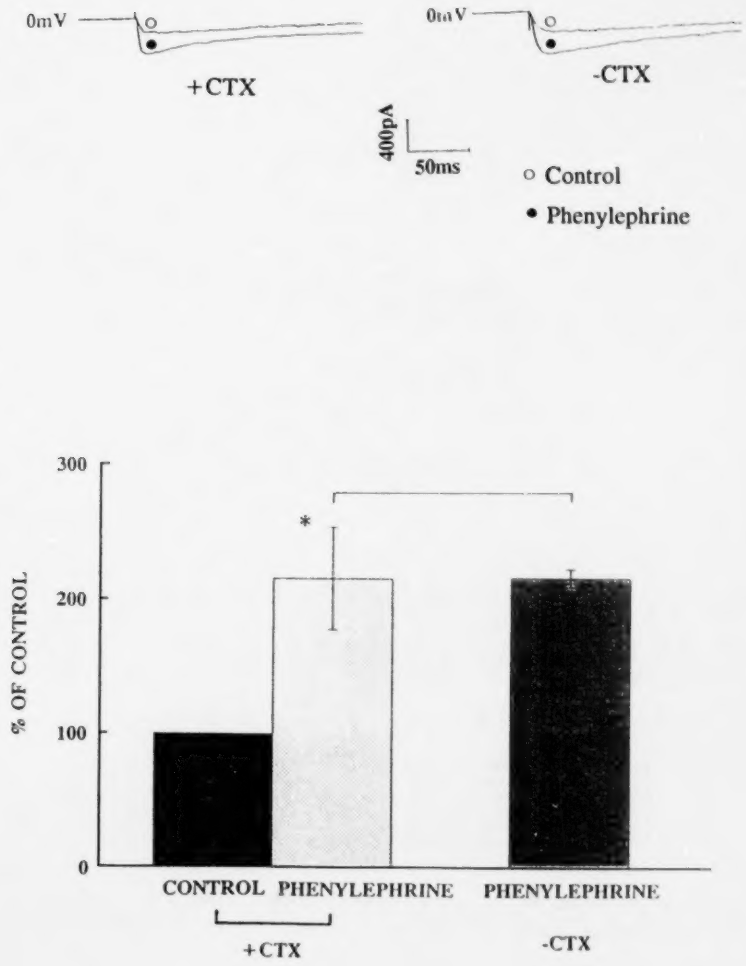


Fig. IV-15. The effect of CTX pretreatment on the phenylephrine-induced increase in Ca^{2+} channel current. Current records from two cells are shown in the upper panel (holding potential of -40 mV). CTX did not affect the phenylephrine-induced increase in L-type current. Experiments using a group of cells confirmed this effect which is shown at the bottom of the figure. Phenylephrine significantly increased L-type current in cells pretreated with CTX (1 μg/ml) (n=5). The magnitude of current increase is the same as that in the absence of pretreatment with CTX (n=7). Values are mean±S.E.M. $^*P<0.05$ (Student's t test).

Fig. IV-16.

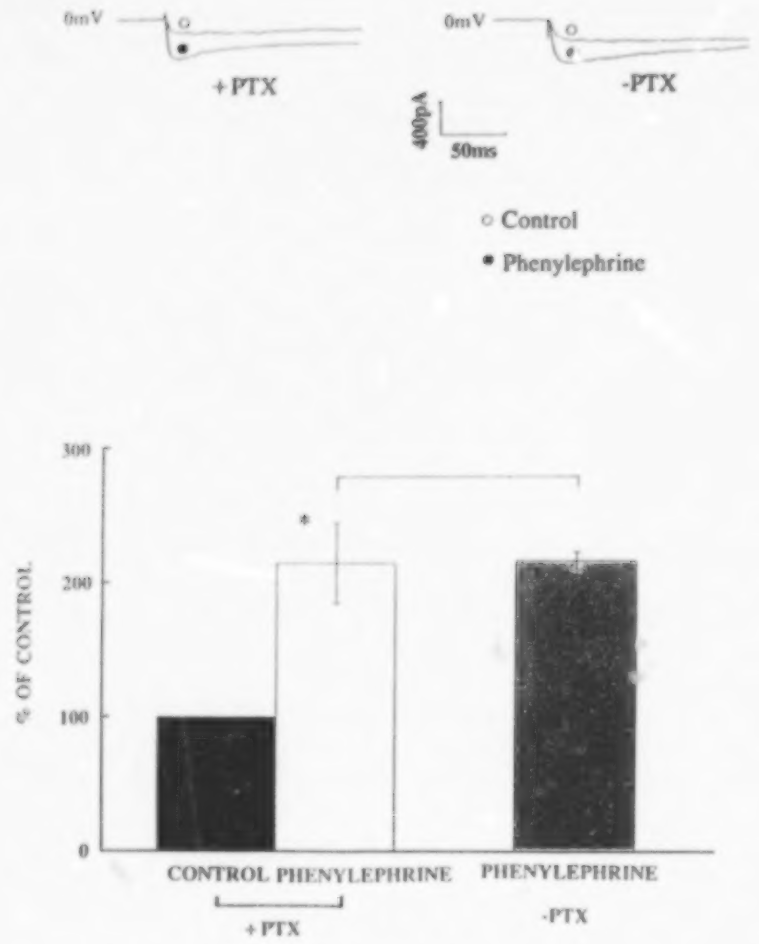


Fig. IV-16. The effect of PTX pretreatment on the phenylephrine-induced increase in Ca^{2+} channel current. The same method was used as described in Fig. IV-14 except that the toxin used was pertussis toxin. PTX pretreatment did not affect the phenylephrine-induced increase in L-type current (n=4).

Fig. IV-17.

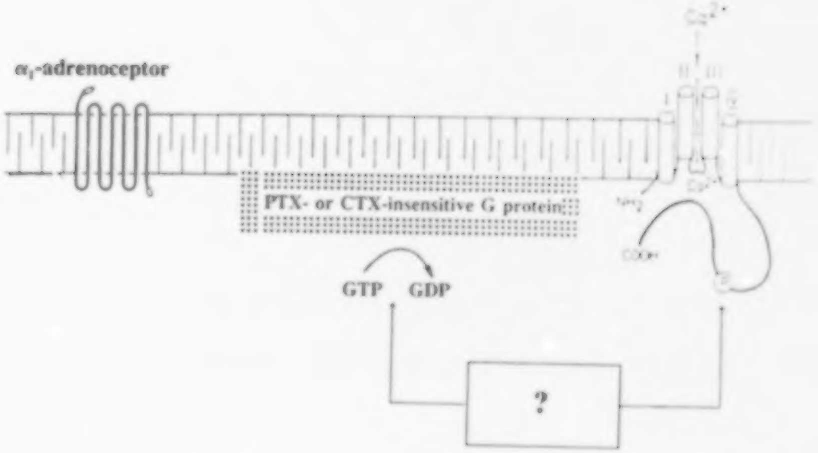

α_1-adrenoceptor
PTX- or CTX-insensitive G protein
GTP
GDP
?
Ca^{2+}
II
III
I
IV
NH_2
Ca^{2+}
COOH
P

Fig. IV-17. Schematic diagram showing that a PTX- and CTX-insensitive G protein mediates the stimulation of the α_1-adrenoceptor-induced increase in Ca^{2+} channel current. The stimulation of the α_1-adrenergic receptor results in G protein activation, which, in turn, results in an increase in L-type Ca^{2+} channel current.

3.3. Effect of Protein Kinases on the Phenylephrine-Enhanced Calcium Channel Current in Neonatal Rat Ventricular Cells

3.3.1. *PKA Did Not Mediate the Phenylephrine-Induced Increase in the L-Type Ca^{2+} Channel Current*

Two agents, S_p-cAMPs (1 mM), a PKA activator, and R_p-cAMPs (100 nM), a PKA inhibitor, were added to the pipette solution in these experiments. These agents were used to determine whether PKA was involved in the mechanism of the phenylephrine-induced increase in the L-type Ca^{2+} channel current. R_p-cAMPs is a cyclic AMP analogue that binds to the R-subunit preventing dissociation of the cAMP holoenzyme (Dewit *et al.*, 1984; Hescheler *et al.*, 1987b). S_p-cAMPs is a cyclic AMP analogue that binds to the R-subunit persistently activating PKA. After access was gained to the interior of the cell, a 10 minute period of intracellular dialysis was allowed. The effect of phenylephrine on the L-type Ca^{2+} channel current was obtained 10 minutes after the addition of phenylephrine to the bath. The results showed that S_p-cAMPs did not potentiate, and R_p-cAMPs did not abolish, the effect of phenylephrine (10^{-5} M), i.e., phenylephrine increased L-type Ca^{2+} channel current in the presence of S_p-cAMPs or R_p-cAMPs (Fig. IV-18). The results obtained from these experiments suggest that the phenylephrine-induced increase in L-type current is not mediated by the cAMP-PKA pathway, as suggested previously (Buxton & Brunton, 1985). The experiments using R_p-cAMP and S_p-cAMP were repeated four times and the same results were observed.

3.3.2. *Protein Kinase C Activation*

Tumour-promoting phorbol esters are known to activate PKC both *in vitro* and in intact cells (Nishizuka, 1984). It has been reported that application of phorbol esters to the bath solution can increase Ca^{2+} channel current in neonatal rat ventricular myocytes (Dosemeci *et al.*, 1988; Lacerda *et al.*, 1988). Therefore, two PKC activators, 4β-phorbol 12-myristate 13-acetate (PMA) (200 nM) and 1-oleoyl 2-acetylglycerol (OAG) (60 μM) were used. PMA, a phorbol ester, caused a significant increase in the L-type Ca^{2+} channel current (Fig. IV-19). Three original current records and the I-V relationship from the same records before and after PMA administration to the bath solution are shown in Fig. IV-19A. The effect of PMA was recorded 5 minutes after administration of PMA to the bath. PMA increased the L-type current, but did not change the channel kinetics. In the five cells tested, 200 nM PMA increased the current amplitude to 159.8 ± 10.6 % (n=5) when compared to the control. The effect of OAG (60 μM), a synthetic membrane permeable DG, is shown in Fig. IV-20. The protocol was the same as that used with PMA. Three original current records and the I-V relationship before and after OAG administration are shown in Fig. IV-20A. Fig. IV-20B shows that 60 μM OAG increased the L-type Ca^{2+} channel current amplitude by 140.6 ± 20.6% when compared with the control values (n=6). The effect of OAG was measured 10 minutes after administration of OAG to the bath. These two agents, PMA and OAG, activate the L-type Ca^{2+} channel current in a different temporal pattern. The effect of PMA reached its peak 2-3 minutes after addition of the drug whereas OAG reached its peak effect 5-10 minutes after addition of the drug (Fig. IV-21). This suggests that PMA may rapidly desensitize Ca^{2+} channels or PKC, which, in turn, results in dephosphorylation of the Ca^{2+} channel proteins while OAG may persistently activate PKC which results in constant activation of the Ca^{2+} channel. The upper panel in Fig. IV-21 shows the original current records at different times before and after exposure to PMA and OAG. The lower panel shows the results from two groups of cells. The currents were normalized to the control current which was taken as 100%. PMA increased the L-type Ca^{2+} channel current in the first 5 minutes. Then the current gradually decreased. Fifteen minutes after exposure to the drug, the current was below the control level (n=5). When OAG was used, the L-type current gradually increased and was maintained up to 15 minutes (n=6). The total Ca^{2+} channel current after activation by OAG or PMA could be completely blocked by 10 μM nifedipine. The total Ca^{2+} channel current consists of the voltage activated current plus the additional PKC activated current. Fig. IV-22 shows that nifedipine completely blocked the total L-type Ca^{2+} channel current enhanced by PMA and generated by depolarization (n=3). Nifedipine also blocked the total current after OAG (data not shown). These results suggest that PMA or OAG (PKC activators), activate dihydropyridine (DHP)-sensitive Ca^{2+} channels in neonatal rat ventricular cells. The enhancement of DHP-sensitive Ca^{2+} channel current by OAG or PMA is similar to the phenylephrine-induced increase in the L-type Ca^{2+} channel current which is also sensitive to DHPs.

3.3.3. *Protein Kinase C Inhibition*

It has been shown that the activation of PKC mimics the effect of the α_1-agonist phenylephrine on the L-type current. However, additional evidence is required to demonstrate that PKC mediates the effect of phenylephrine on the Ca^{2+} channel current. Therefore, two PKC inhibitors were used to abolish the effect of phenylephrine on the L-type current. Staurosporine (100 nM), a microbial alkaloid found to be a potent inhibitor of PKC, was used in these experiments (Tamaoki *et al.*, 1986). Staurosporine did not show any effect on the L-type Ca^{2+} channel current 5 minutes after the cells were exposed to the drug (Fig IV-23). The I-V relationship 5 minutes after the addition of staurosporine was generated and used as the control. The effect of phenylephrine was measured 10 minutes after addition of staurosporine to the bath. The phenylephrine-induced increase in the L-type Ca^{2+} channel current was completely abolished by staurosporine (Fig. IV-24), indicating that PKC might play an obligatory role in the phenylephrine-induced increase in the L-type current. Fig. IV-24A shows the original current records and the I-V relationship from the same records before and after staurosporine followed by phenylephrine. Fig. IV-24B shows that staurosporine completely abolished the effect of phenylephrine on the L-type current in a group of cells. The histogram was generated by normalizing the current after phenylephrine addition to the control current and the data are plotted as a percentage of the control. The normalized current induced by phenylephrine (10 μM) after staurosporine was 99.5±10.6% which was significantly different from the current after phenylephrine in the absence of staurosporine (206.0±20.7%). Therefore, staurosporine abolished the phenylephrine-induced increase in L-type Ca^{2+} channel current.

An alternative approach which may be used to block endogenous PKC activation is pre-incubation of cells with 500 nM PMA for 7-9 hours. This down-regulates the levels of the endogenous enzyme (Braun *et al.*, 1990). It is believed that prolonged stimulation of PKC by phorbol esters leads to a loss of the enzyme as a result of proteolytic degradation. Henrich and Simpson (1988) have shown that pre-treatment of cultured neonatal rat cardiac myocytes with 100 nM PMA virtually eliminated total cellular PKC activity and specific phorbol ester binding sites after 6 hours of incubation. Therefore, 500 nM PMA was used to pretreat the neonatal rat ventricular cells for 7-9 hours. These results show that after pretreatment with PMA, the phenylephrine-induced increase in L-type current was almost completely abolished (Fig. IV-25). Fig. IV-25A shows two current records and the I-V relationship before and after phenylephrine in the cells pretreated with PMA for 7-9 hours. Fig. IV-25B shows that PMA pretreatment blocked the phenylephrine-enhanced L-type current in a group of cells. The normalized current amplitude after phenylephrine in the group of cells pretreated with PMA was 114.6±23.4% (n=4) when compared to the control values. It is clear that without PMA pretreatment of the cells, the L-type current was increased by phenylephrine (10^{-5} M) to 206.0±20.7% (n=9) which is significantly different from that of the cells with PMA pretreatment. Therefore, these results confirm that PKC plays an obligatory role in the mediation of the α_1-adrenoceptor modulation of the L-type Ca^{2+} current in neonatal ventricular cells. This is shown in the schematic diagram (Fig. IV-26).

Fig. IV-18.

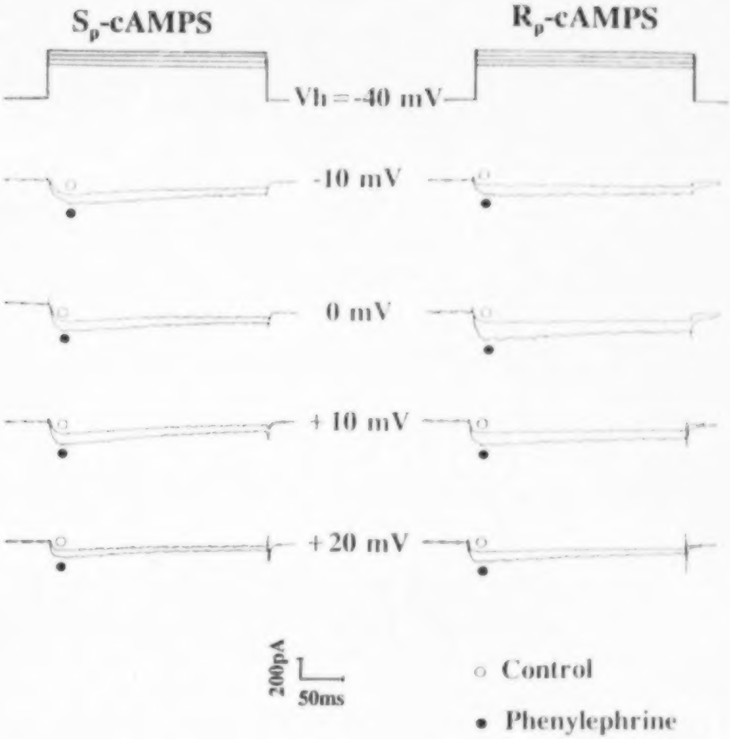


Fig. VI-18. Effects of a PKA activator and inhibitor on the phenylephrine-induced increase in the L-type Ca^{2+} channel current. The original current records from two cells are shown. The cells were depolarized from a holding potential of -40 mV to various test potentials indicated next to each record. One cell was dialysed with 1 mM S_p-cAMPs, a PKA activator, and the other with 100 nM R_p-cAMPs, a PKA inhibitor. After a 10 min period of intracellular dialysis, subsequent addition of phenylephrine resulted in an increase in the L-type Ca^{2+} channel current.

Fig. IV-19.

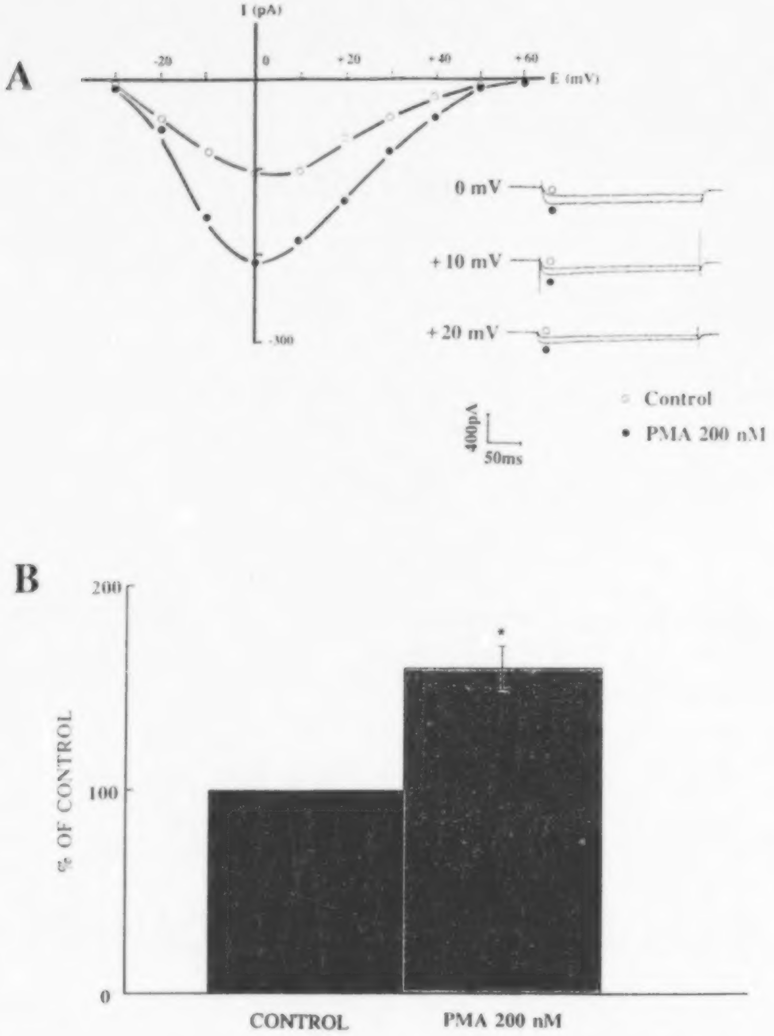


Fig. IV-19. Effects of the PKC activator PMA (200 nM) on the L-type Ca^{2+} channel current. (A). I-V relationship and three original records from the same cell before and after addition of PMA. PMA increased the L-type Ca^{2+} channel current but did not shift the peak amplitude along the voltage axis. (B). 200 nM PMA significantly increased the L-type current in a group of cells (n=5). Values are expressed as mean±S.E.M. $^*P<0.05$ (Student's t test).

Fig. IV-20.

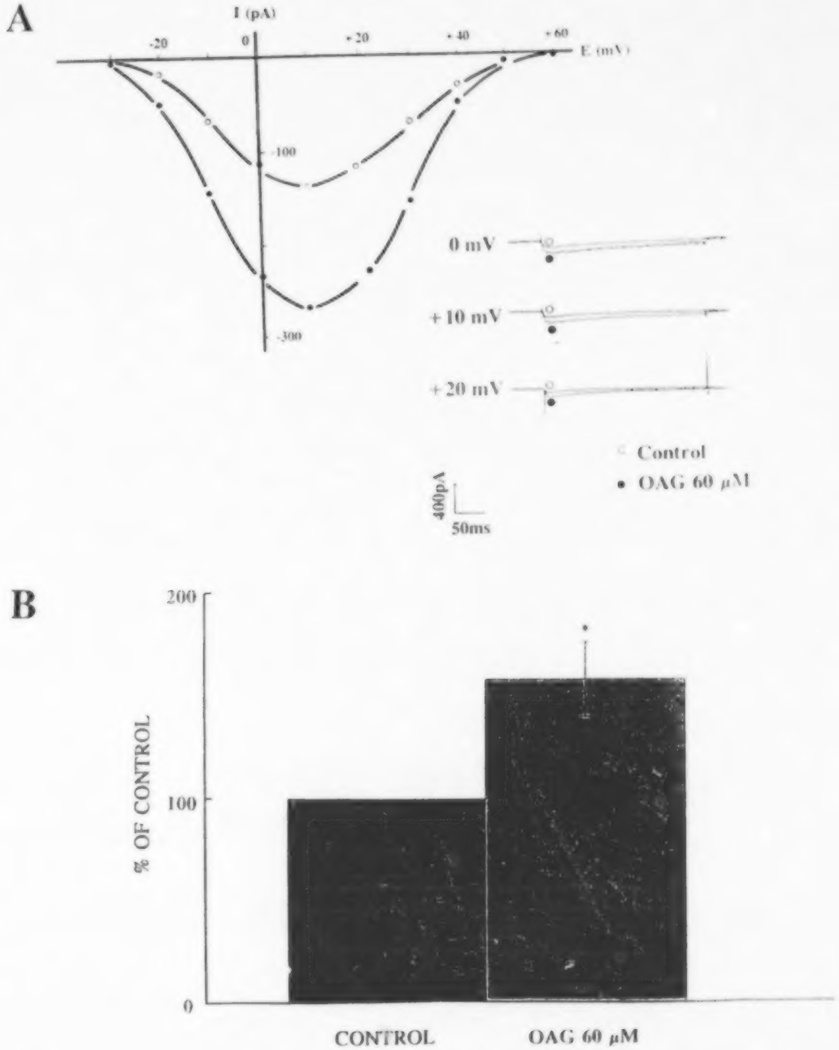


Fig. IV-20. Effect of the PKC activator, OAG (60 μM), on L-type Ca^{2+} channel current. (A). I-V relationship and three original records from the same cell before and after addition of OAG. OAG increased L-type current but did not shift the peak amplitude along the voltage axis. (B). 60 μM OAG significantly increased the L-type current in a group of cells (n=6). Values are expressed as mean ± S.E.M. *$P<0.05$ (Student's t test).

Fig. IV-21.

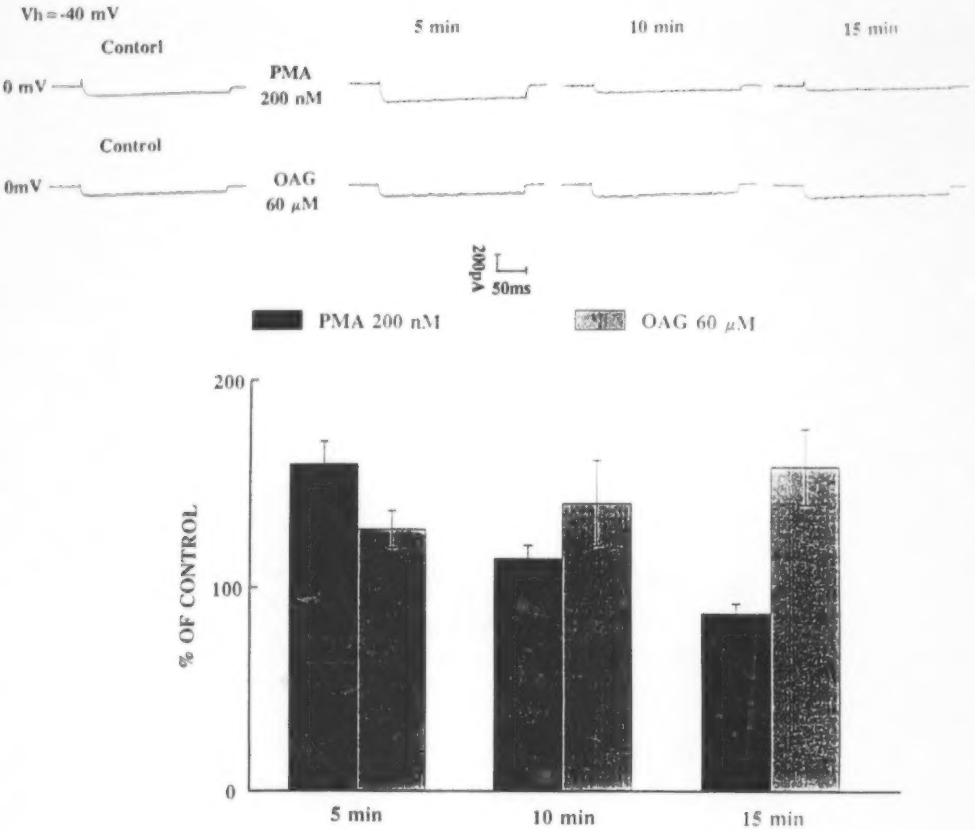


Fig. IV-21. Comparison of the effect of two PKC activators, PMA (200 nM) and OAG (60 μM). The L-type current at 5, 10, 15 and 20 min. Upper panel: the original current records from two cells. The currents were activated by depolarizing the cells to 0 mV from a holding potential of -40 mV. PMA first increased the L-type Ca^{2+} current then decreased it whereas OAG progressively activated the L-type Ca^{2+} channel current over 15 min. The lower panel shows the same results from a group of cells. Values are expressed as percentage of control, mean±S.E.M. PMA reached its peak effect on the L-type current at 2-3 min and then the current gradually decreased. Fifteen min after addition of the drug, the current amplitude was lower than the control (n=5). OAG reached its peak effect on the L-type current 5-10 min after addition of the drug and this effect was maintained up to 15 min (n=6).

Fig. IV-22.

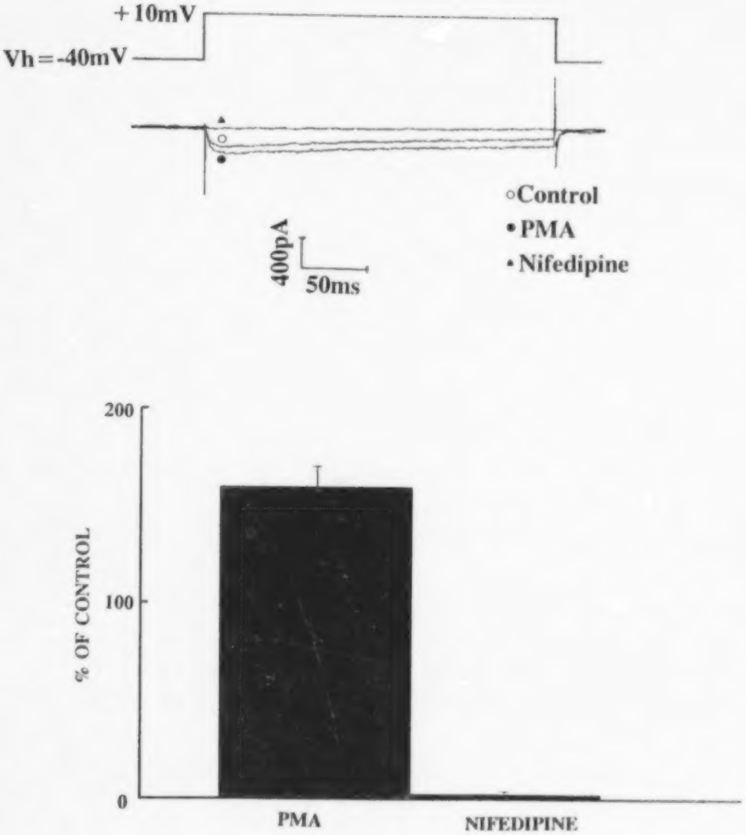


Fig. IV-22. The effect of nifedipine (10 μM) on the total current (see text) after treatment with PMA. The upper panel shows that the total current was completely blocked by nifedipine. The lower panel shows these results in a group of cells. Nifedipine completely blocked the L channel current including PMA enhanced-current from a group of cells and the current generated by depolarization (n=3). Values are expressed as percentage of control (mean±S.E.M.). *$P<0.05$ (Student's t test).

Fig. IV-23.

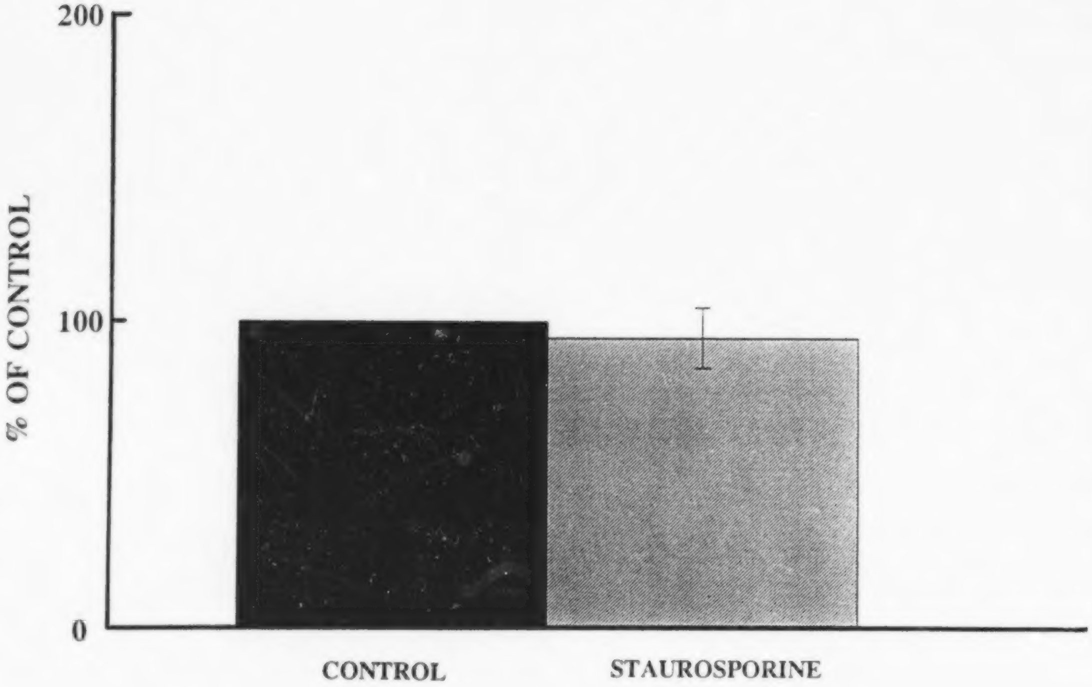


Fig. IV-23. Effect of staurosporine (100 μM) on the L-type Ca^{2+} channel current. Staurosporine, a PKC inhibitor, had no effect on the L-type current. Values are expressed as percentage of control, mean±S.E.M. (n=4).

Fig. IV-24.

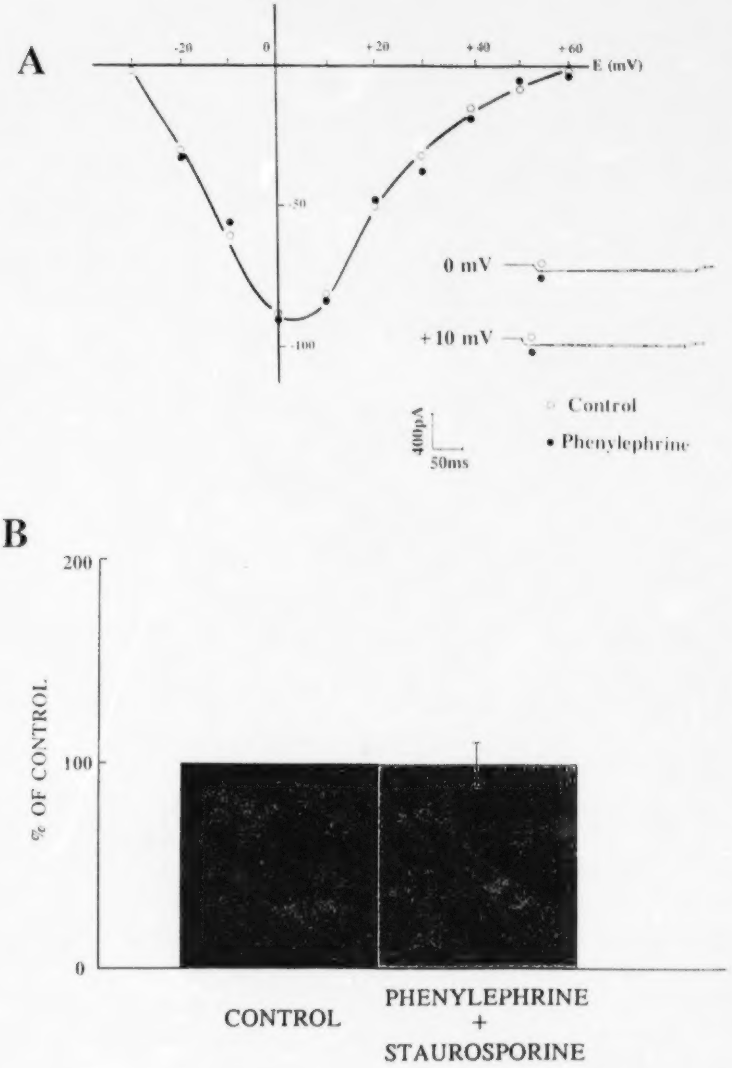


Fig. IV-24. Effect of staurosporine (100 μM) on phenylephrine-enhanced L-type current. (A). I-V relationship and two original current records from the same cell before and after addition of staurosporine followed by phenylephrine. (B). Staurosporine completely abolished the effect of phenylephrine on the L-type current in a group of cells (n=4). Values are expressed as percentage of control (mean±S.E.M.).

Fig. IV-25.

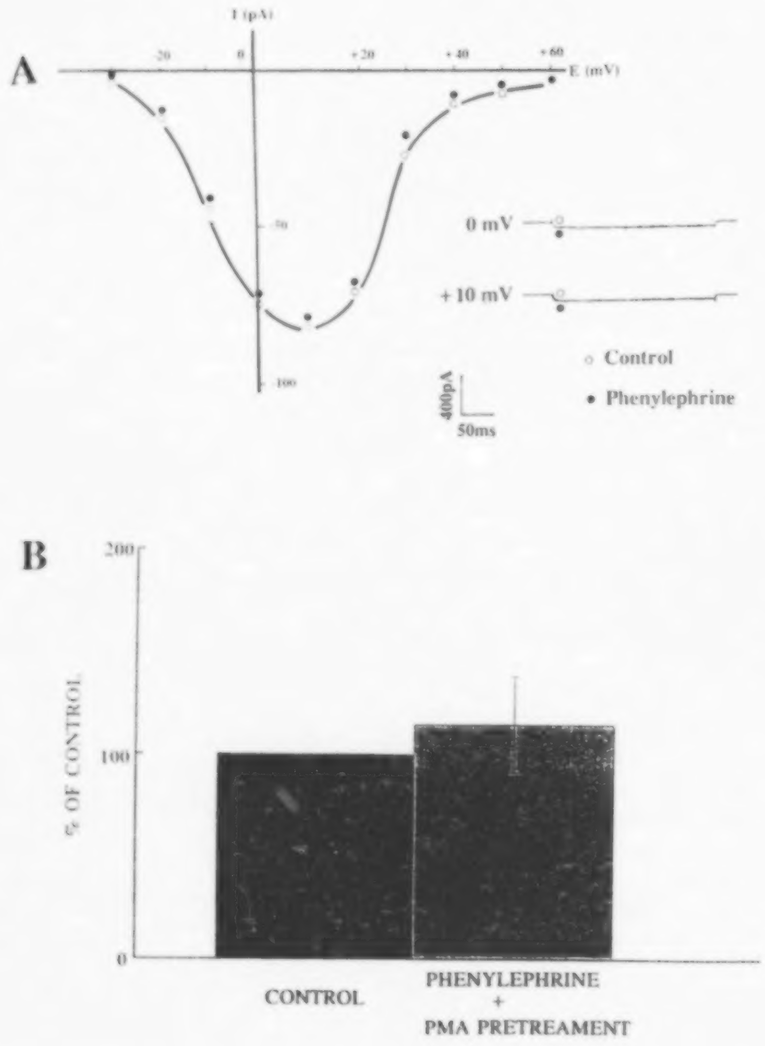


Fig. IV-25. Effect of PMA (500 nM) pretreatment of cells on the phenylephrine-enhanced L-type current. (A). I-V relationship and two original current records before and after addition of phenylephrine in a cell pretreated with PMA for 9 hours. (B). The effect of phenylephrine on L-type current was blocked by pretreatment with PMA (n=4). Values are expressed as percentage of control, and plotted as mean±S.E.M. $P>0.05$ (Student's t test).

Fig. IV-26.

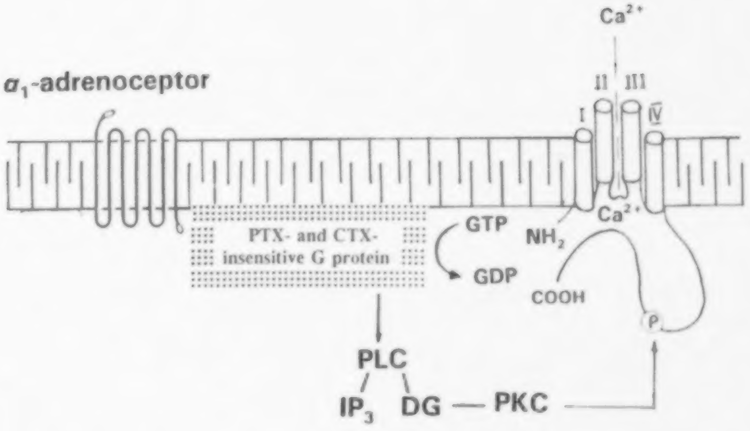

Ca2+
II III
I
IV
α1-adrenoceptor
PTX- and CTX-insensitive G protein
GTP
GDP
NH2
COOH
Ca2+
P
PLC
IP3
DG — PKC

Fig. IV-26. Schematic diagram showing that stimulation of the α_1-adrenoceptor activates a PTX- and CTX-insensitive G protein which, in turn, results in the hydrolysis of PIP_2 which releases IP_3 and activates PKC. PKC phosphorylates the L-type Ca^{2+} channel proteins (DHP-sensitive channel) which results in the activation of the channels.

4. Discussion

The positive inotropic effects of α-adrenergic agonists on cardiac muscle of many species are well documented (Benfy, 1980; Brükner *et al.*, 1985; Schümann *et al.*, 1987) The response to α agonists consists of two phases, a transient phase and a delayed sustained positive inotropic phase. The transient inotropic phase may be mediated by elevated IP_3 which results in intracellular Ca^{2+} release (Otani *et al.*, 1986; Poggioli *et al.*, 1986). The sustained response may be related to protein kinase C activation and involves voltage-gated Ca^{2+} channels (Otani *et al.*, 1988; Hartzell, 1989). The results presented in this study show that phenylephrine increased tension in driven neonatal rat ventricles and that this increase in tension was concentration-dependent. Since these measurements represent a steady-state determination, the results obtained correspond to the sustained phase. Corresponding to the increase in tension, an increase in the plateau phase of the ventricular action potential was also demonstrated in dissociated cells. The increase in ventricular tension and change in the action potential may be attributed to an increase in the L-type Ca^{2+} channel current. However, other possible mechanisms, such as a reduction of I_t, may also be involved in the prolongation of the plateau phase of action potential and tension increase.

The results show that the effect of the α_1-agonist phenylephrine on the L-type current was concentration-dependent. The response to phenylephrine was slow which was consistent with the report of Hartzell (1989), suggesting that the sustained inotropic response requires several minutes to develop. The effect of phenylephrine on the Ca^{2+}

current was not easily reversible by washout. This suggests that phenylephrine does not directly act on the Ca^{2+} channel proteins, and that the effect is mediated by G proteins (Trautwein & Hescheler, 1990) which are coupled to α_1-receptors and intracellular enzymes. It was also observed that 10 μM nifedipine completely blocked the total inward current. The total current is made up of a depolarization evoked component and the additional current generated by phenylephrine modulation. This was in agreement with previous reports that nifedipine reduced the sustained response to α_1-agonists (Tung *et al.*, 1983; Rand *et al.*, 1986; Otani *et al.*, 1988). Dihydropyridine sensitivity indicates that the L-type Ca^{2+} channels are involved.

The effect of phenylephrine on the L-type current was more prominent in the negative range of potentials, which has also been demonstrated for the β-adrenergic agonist isoprenaline (Bean *et al.*, 1984). It has been shown that the enhancement of the Ca^{2+} channel current was greatest at weak depolarizations and smallest at more positive potentials. This may be attributed to the phosphorylation of the Ca^{2+} channel protein, which results in a change in channel gating and, in turn, additional Ca^{2+} channels are open at more negative potentials (Josephson & Sperelakis, 1991). The L-type current in these cells with Ba^{2+} as the charge carrier shows little or no inactivation. However, after phenylephrine, the inactivation of the current was more apparent. Phenylephrine may facilitate opening at more negative potentials and prolong the mean open time of the channel as is the case with Bay K 8644 (Hess *et al.*, 1984). It is also of interest that phenylephrine shifted the steady-state activation and inactivation curves towards more negative potentials, as is the case with Bay K 8644. The effects of phenylephrine probably result in an increase in more functional channels due to phosphorylation and also a change in the mean open time of the active channels (Bean *et al.*, 1984). Unfortunately, the results from this study do not allow one to draw any concrete conclusions. In contrast to its effects on the L channel current in this preparation, phenylephrine had no effect on the T channel current. However, it has been reported that in 70% of canine Purkinje cells, α-adrenoceptor stimulation by 10 μM norepinephrine (in the presence of 2 μM propranolol) increased the T-type Ca^{2+} channel current (Tseng & Boyden, 1989).

It has been suggested that whether phenylephrine exerts inotropic effects through α- or β-adrenoceptors depends upon the relative receptor populations (Chess-Williams *et al.*, 1990) and there is considerable species variability in the positive inotropic response to α-agonists (Wagner & Brodde, 1978). The α_1-adrenergic receptor density in rat heart was the highest when compared with that of baboon, calf or dog. The density of the receptors is usually associated with major physiological differences between these species in response to phenylephrine (Shen *et al.*, 1989). The density of the adult rat ventricular α_1-adrenoceptors was four times greater than β-adrenoceptor density. This was shown using the [^{3}H]-prazosin and [^{3}H]-propranolol binding assay (Chess-Williams *et al.*, 1990). Furthermore, it has been reported that neonatal rat ventricular myocytes expressed predominantly α_1-adrenoceptors rather than β-adrenoceptors (Karliner *et al.*, 1985). The results described in this report show that the enhancement of the L-type current induced by phenylephrine can be totally abolished by the application of an equimolar concentration of the α_1-antagonist prazosin. The α_2-adrenergic agonist clonidine and the antagonist yohimbine were also used to eliminate the possible effect of the α_2-adrenoceptor on the Ca^{2+} channel current. These results are consistent with other reports that the α_1-adrenoceptor is the predominant α-receptor in the heart (Buxton & Brunton, 1986). It has been reported that the cardiostimulatory effect of phenylephrine on isolated atria of the guinea-pig and rabbit was mediated by β-adrenoceptors (Benfy & Carolin, 1971; Wagner & Reinhardt, 1974). In order to exclude the possibility that phenylephrine activates β-adrenoceptors in these experiments, the β-antagonist propranolol was applied prior to phenylephrine administration and the effect of phenylephrine on the L-type current was still observed in all cells tested. Therefore, it is clear that the phenylephrine effect on the L-type current is mediated exclusively by the α_1-adrenoceptor in neonatal rat myocytes.

Guanine nucleotides have been shown to exert regulatory effects on the stimulation of receptors which result in the activation of adenylate cyclase and other biochemical effects. They are also required for receptor-activated transduction mediated by phosphatidylinositol hydrolysis and ion channel modulation (Spiegel, 1987). The non-hydrolysable guanine nucleotide analogues GTPγS and GDPβS are useful probes for determining G-protein involvement. The reasons are as follows. 1) They can substitute for endogenous GTP in G protein binding. 2) These compounds are a poor substrate for intrinsic GTPase activity of the G protein and, thus, their effects are persistent. 3) They can cause G protein activation in the absence of agonist-receptor interaction (Gilman, 1987; Schofield, 1991). GDPβS, a G protein inhibitor, completely blocks the phenylephrine-induced increase in the L-type Ca^{2+} channel current and GTPγS, a G protein activator, increases the phenylephrine-induced increase in L-type Ca^{2+} channel current. These results support the conclusion that a G protein is involved in the phenylephrine modulation of the L-type Ca^{2+} channel current.

The family of G proteins that serves as pertussis toxin and cholera toxin substrates has several members. All these substrates have ADP-ribosylatable α subunits that are similar in molecular weights for each toxin (Gilman, 1987; Moss, 1987; Spiegel, 1987). The neonatal rat heart contains three G protein substrates (45-, 47- and 52- KD_a) for CTX-catalyzed ADP-ribosylation and there is one G protein substrate (41 KD_a) for PTX-catalyzed ADP-ribosylation (Kojima *et al.*, 1988). Although the existence of substrates for PTX and CTX is evident, the results in this thesis demonstrated that the α_1-adrenergic agonist phenylephrine-induced increase in the L-type Ca^{2+} channel currents is not affected by these two toxins. It is possible that the G protein involved in this α_1-adrenergic response in neonatal rat heart is a toxin-insensitive G protein. This hypothesis was supported by other reports that the positive inotropic effect mediated by α_1-adrenoceptors does not involve a PTX-sensitive G protein (Böhm *et al.*, 1987; Schmitz *et al.*, 1987). Recently, two novel murine cDNA clones, α_q and α_{11}, were isolated (Strathmann & Simon, 1990). These two α subunits represent a new class of G proteins, G_q, which has the ability to activate phospholipase C (Pang & Sternweis, 1990; Smrcka *et al.*, 1991). The α_q and α_{11} lack the cysteine residue four amino acids from the carboxyl terminal that serves as a target for covalent modification by PTX (Shenker *et al.*, 1991). Thus, it is suggested that a PTX-insensitive G protein activates PLC which results in the hydrolysis of PIP_2 into IP_3 and DG. The results presented in this chapter provide direct evidence of a linkage of a pertussis toxin-insensitive G protein to the α_1-adrenergic receptor in the neonatal rat ventricular cells, which is in agreement with a previous report (Steinberg, 1987). Unfortunately, these studies do not permit specific identification of the G protein.

Several groups of investigators have provided evidence that the α_1-adrenergic receptor is coupled to a G protein in cardiac tissue (Colucci *et al.*, 1984; Buxton & Brunton, 1985; Drugge *et al.*, 1985; Han *et al.*, 1989). It has been reported that primary cultures of neonatal rat ventricular myocytes have a positive chronotropic response. This chronotropic response cannot be blocked by PTX (Han *et al.*,1989), but is associated with IP_3 formation (Steinberg *et al.*, 1987, 1989). Therefore, the involvement of a PTX-insensitive G protein was suggested in the α_1-adrenoceptor-induced positive chronotropic response in neonatal rat ventricular myocytes.

The best studied example of a hormonal effect on the calcium channel current is the β-adrenergic stimulation of the L-type Ca^{2+} channel in heart (Reuter, 1983; Tsien *et al.*, 1986; Yatani & Brown, 1989). Agonists acting on β-adrenoceptors activate the stimulatory G protein (G_s), which, in turn, activates the adenylate cyclase, elevating intracellular cAMP. The cAMP acts on the cAMP-dependent protein kinase which phosphorylates Ca^{2+} channel protein and this leads to an increase in I_{Ca} (Trautwein & Hescheler, 1990). Therefore, G_s serves as a transducer converting the signal from the outside to the inside of the cell. Several G proteins have been found to be involved in the modulation of Ca^{2+} channels. In neuronal tissue, G proteins mediate the inhibition of Ca^{2+} channel current (Holz *et al.*, 1986). In endocrine secretory cells, a G_i-type of G protein mediates both the stimulation and the inhibition of Ca^{2+} current (Rosenthal *et al.*, 1988a). In cardiac myocytes, G_s activates Ca^{2+} currents. However, there are no studies demonstrating that a PTX-insensitive G protein modulates VDCCs. In these experiments, the involvement of a G protein in the modulation of the L-type Ca^{2+} channel current was studied using GTPγS and GDPβS. Furthermore, PTX and CTX were used to rule out the toxin sensitive G proteins. This is the first report describing a PTX- and a CTX-insensitive G protein which modulates the L-type VDCC.

The activation of adenylate cyclase can result from the activation of G_s. This leads to the stimulation of a cAMP-dependent kinase which, in turn, increases cardiac Ca^{2+} channel currents (Schultz *et al.*, 1990). Recent evidence suggests that G_s also activates Ca^{2+} channels independently of a cAMP-dependent step. In isolated inside-out patches of cardiac myocytes and in cardiac sarcolemma or T tubule membranes incorporated into phospholipid bilayers, various forms of the G_s α-subunit activate Ca^{2+} channels if applied to the cytoplasmic side (Yatani *et al.*, 1987a; Imoto *et al.*, 1988; Mattera *et al.*, 1989). Thus, G_s controls Ca^{2+} channels by a membrane-delimited pathway. It has been shown that the modulation of Ca^{2+} channel current by G proteins through second messengers appears to be slow while the membrane-delimited pathway appears to be fast (Yatani & Brown, 1989). Since the phenylephrine-induced increase in I_{Ca} is slow, it suggested that this is mechanism is mediated by G proteins and a second messenger pathway. However, the data in this thesis cannot exclude the possibility that the G proteins also modulate Ca^{2+} channels directly.

Two protein kinases, PKA and PKC, have been shown to phosphorylate DHP-sensitive Ca^{2+} channels (Chang *et al.*, 1991; Gutierrez *et al.*, 1991). These two protein kinases were studied in this thesis to determine whether they participate in the α_1-adrenoceptor induced L-type Ca^{2+} channel current increase. The data indicate that the cAMP-PKA pathway did not play a major role in this mechanism. In agreement with the above results, it has been reported that in isolated rat myocytes and in isolated perfused rat heart, the activation of α_1-adrenergic receptors with phenylephrine in the presence of propranolol resulted in a reduction in cAMP levels (Watanabe *et al.*, 1977). This decrease in cAMP was due to the activation of a cAMP phosphodiesterase. Thus, the cellular cAMP rapidly hydrolysed to AMP (Buxton & Brunton, 1985). Since the G protein involved in α_1-adrenoceptor activation was not G_s or G_i as demonstrated in section II, protein kinase A can be excluded as a mediator of the α_1-adrenoceptor activation-induced L-type current increase.

Calcium translocation produced by agonist interaction with α_1-adrenoceptors is believed to be secondary to enhanced phosphatidylinositol (PI) turnover, due to activation of phospholipase C, in the "phenylephrine response" (Michell, 1979; Nichols, 1991). It appears that the α_1-adrenoceptor-mediated responses are the result of an increase in PI turnover in many tissues (Woodcock *et al.*, 1987; Steinberg *et al.*, 1989). This response results in the generation of IP_3 and DG (Berridge *et al.*, 1986), both of which have been shown to posses second messenger functions within cells. IP_3 elicits a rapid release of calcium from intracellular, non-mitochondrial stores whereas DG is an activator of protein kinase C. PKC has been shown to modulate Ca^{2+} channels in various tissues (Shearman *et al.*, 1989). In neonatal rat ventricular cells, phorbol ester, a PKC activator, stimulates dihydropyridine-sensitive $^{45}Ca^{2+}$-influx and this effect can be reversed by a 20 minute pre-incubation of the cells with phorbol ester (Lacerda *et al.*, 1988). There is evidence that the L-type Ca^{2+} channel current was increased by phorbol ester (Dosemrci *et al.*, 1988). Therefore, according to the literature, it was very tempting to draw the conclusion that the DG-PKC pathway, one of the PIP_2 bifurcating pathways, may mediate α_1-adrenoceptor stimulation-induced L-type Ca^{2+} channel current increase. However, contradictory data suggest that α_1-agonists and PMA produced very different effects on cardiac function, such as ventricular contraction. PMA was reported to activate PKC to a greater extent and more persistently than norepinephrine in neonatal rat myocytes (Henrich & Simpson, 1988). Other possible mechanisms may also be involved in the activation of PKC such as unsaturated fatty acids and the elevatation of $[Ca^{2+}]_i$.

It was also demonstrated that PKC activators could not mimic the electromechanical response mediated by α_1-adrenoceptors in adult rat and rabbit atrial myocytes, thus suggesting that activation of PKC may not play an important role in the positive inotropic response (Braun *et al.*, 1990; Tohse *et al.*, 1990). In contrast, evidence has revealed that stimulation of protein kinase C using phorbol ester does have an effect on cardiac muscle contraction (Hartzell, 1989). According to Otani *et al.* (1988), α_1-adrenoceptor stimulation resulted in a triphasic inotropic response. A transient increase within 30 seconds was followed by a transient decline in the contractility prior to the development of a sustained and more pronounced positive inotropic response. The sustained positive inotropic response (PIE) was proposed to be caused by increased Ca^{2+} influx via slow Ca^{2+} channels and this sustained PIE may be mediated by DG through activation of PKC (Otani *et al.*, 1988). Nevertheless, the results from this thesis show that PKC activators OAG and PMA mimicked the effect of phenylephrine on the L current. The PKC inhibitor staurosporine and depletion of PKC activity using prolonged PMA-pretreatment blocked the effect of phenylephrine on the L-type Ca^{2+} channel current, suggesting that PKC indeed mediated the α_1-adrenoceptor-induced increase in L-type current in neonatal rat ventricular cells.

The studies established clearly that α_1-adrenergic stimulation-induced L-type Ca^{2+} channel current increase is mediated by a PTX- and CTX-insensitive G protein and followed by PKC activation. The PTX-insensitive G protein (G_q or G_{11}) was found to generate PIP_2 hydrolysis (Smrcka *et al.*, 1991; Waldo *et al.*, 1991). This G protein has not been identified in neonatal rat myocytes. However, other evidence has shown that a PTX-insensitive G protein might exist, be coupled to an α_1-adrenoceptor, and elicit hydrolysis of PIP_2 (Steinberg *et al.*, 1989). Several mechanisms are now also recognized to be regulated by cardiac α_1-adrenergic receptors. These include a K^+ current (Apkon & Nerbonne, 1988), Na-K ATPase (Shah *et al.*, 1988), guanylate cyclase (Vulliemoz *et al.*, 1987) and inhibition of β-adrenergic dependent cyclic AMP generation (Barrett *et al.*, 1987). Here, a new mechanism is reported which is different from other known mechanisms. The discrepancy among these mechanisms might be due to tissue differences. Nevertheless, this is the first time that cardiac α_1-adrenergic dependent Ca^{2+} channel activation mediated by a PTX-insensitive G protein and PKC has been reported.

5. Summary

The results presented in this chapter describe a second messenger pathway in which a PTX- and CTX-insensitive G protein couples the α_1-adrenoceptor to the L-type Ca^{2+} channel current through PKC. The cells used in this study were 3 day old neonatal rat ventricular cells. This is the first report to show that stimulation of the α_1-adrenoceptor in the neonatal rat heart results in an increase in the L-type Ca^{2+} channel current. These results are contradictory to reports using adult animals (Apkon & Nerbonne, 1988; Fedida *et al.*, 1991). The discrepancy may be attributed to differences during development. The enhanced Ca^{2+} channel current induced by the α_1-adrenergic agonist phenylephrine was consistent with the studies of increased ventricular tension and prolonged plateau phase of the action potential using neonatal preparations. The effect of phenylephrine on the L-type Ca^{2+} channel current was exclusively through α_1-adrenoceptors since α_2-adrenergic agonists did not elicit the same effect as did phenylephrine, and α_2, β-adrenergic antagonists did not block the phenylephrine effect. Thus, it is concluded that α_1-adrenoceptor activation is indeed responsible for the L-type Ca^{2+} channel current increase in neonatal rat ventricular cells. This effect was observed to be slow and to persist during the expermintal period. G protein and the second messenger cascades are likely to be involved in the α_1-adrenergic response since a multiple element system is slow, as suggested by Brown (1991). With the help of GTPγS and GDPβS, the evidence of G protein involvement in the α_1-adrenergic agonist-induced L-type Ca^{2+} channel current and the toxin studies suggested that the G protein involved in this response was PTX- and CTX-insensitive. After G protein activation, intracellular second messengers are activated to interact with Ca^{2+} channel proteins. It is widely accepted that protein kinases phosphorylate Ca^{2+} channel proteins and make them open or close (Reuter *et al.*, 1986). The α_1-adrenergic response in the heart has been reported to be associated with PIP_2 hydrolysis. Thus, PKC might be responsible for this phenylephrine effect. The results clearly showed that PKC plays an obligatory role in the phenylephrine-induced increase in Ca^{2+} current, while PKA is not involved. In this chapter, it has been shown that the modulation of Ca^{2+} channels by an α_1-adrenoceptor agonist causes an increase in Ca^{2+} current in intact cardiac cells. This occurs through a cascade of events, finally leading to a PKC-dependent phosphorylation of one of the subunits of the channel protein (Curtis & Catterall, 1985).

Under normal physiological conditions, the cardiac α_1-adrenoceptor may be unimportant in cardiac contractility. However, an altered α-adrenergic receptor may be of critical importance in the pathogenesis of arrhythmias, influencing myocardial oxygen demand and myocardial cell viability after an ischaemic insult (Kagiya *et al.*, 1991). It was found that the number of α_1-adrenoceptors increase in response to hypoxia in neonatal rat myocytes and ischaemic cat and guinea pig myocardium (Heathers *et al.*, 1987; Maisel *et al.*, 1987; Kagiya *et al.*, 1991). Furthermore, the α-adrenergic receptor system may exist as a secondary or reserve inotropic system in the heart if the β-adrenergic receptors become unresponsive (Corr *et al.*, 1990). In addition, α-adrenergic receptors and G proteins are of importance in terms of development, since the density of the receptors and their coupling system change with age. Therefore, this study of α_1-

adrenergic receptor modulation of Ca^{2+} channels may provide information with respect to the developmental changes of ventricular cells. This, in turn, may also be important in the pathogenesis of arrhythmias and ischemia.

CHAPTER V. THE EFFECTS OF PHENYLEPHRINE ON CALCIUM CHANNEL CURRENT IN ADULT RAT VENTRICULAR CELLS

1. Introduction

It has been suggested that the positive inotropic response to α_1-adrenoceptor activation was mediated by the reduction of K^+ current and not associated with Ca^{2+} channel currents in adult rat heart (Apkon & Nerbonne, 1988; Ravens *et al.*, 1989). The purpose of the present study was to compare the effect of α_1-adrenergic stimulation on Ca^{2+} channel currents in neonatal and adult rat ventricular myocytes by investigating the effect of the α_1-adrenergic agonist phenylephrine on L-type Ca^{2+} channel currents in adult rat ventricular cells. The experimental conditions used were similar to those used for studies of neonatal rat ventricular cells.

2. Experimental Design

Freshly dissociated adult ventricular cells were used in the experiments. The L-type Ca^{2+} channel current was identified and the effect of phenylephrine on L-type Ca^{2+} channel current in these cells was determined using electrophysiological and pharmacological techniques.

3. Results

A size comparison of neonatal and adult rats is given in Fig. V-1 (top). Neonatal rats were 3 to 4 day-old and weighed 8-12 g. The young adult rats used for the comparative study weighed 150-200 g. The ventricular myocytes from neonatal and adult rats were also compared in Fig. V-1 (bottom). The myocytes from neonatal rat heart were dissociated using trypsin and incubated overnight. The myocytes from adult rat heart were dissociated using collagenase and were used for electrophysiological recording 2 hours after dissociation. The ventricular myocytes from neonatal rats were round in shape with a diameter of 24 μm, while the ventricular myocytes from adult rats were rod shaped, 84-120 μm in length and 20-24 μm in width. The cross striations can be clearly seen in cells from the adult rat. Both types of cells contracted in response to various stimuli.

Electrophysiological recordings were performed on freshly dissociated adult rat ventricular myocytes. From a holding potential of -40 mV, the currents activated by depolarizing a cell to various test potentials are shown in Fig. V-2. The currents inactivated by approximately 70% during the 400 ms duration depolarization. The peak amplitudes of the currents were 1-1.5 nA. The channel was activated at -20 mV and the maximum current occurred at 0 or +10 mV. This current was completely blocked by nifedipine (10 μM), indicating this is an L-type Ca^{2+} channel current (Bean, 1989) (Fig. V-3). The effect of phenylephrine (10 μM) on the L-type Ca^{2+} channel current is shown in Fig. V-4. Phenylephrine did not affect the L-type current during a 30 minute experiment.

However, Bay K 8644 (1 μM) increased L-type current and La^{3+} (2 mM) completely blocked this current. The upper panel in Fig. V-4. shows three current records obtained from an adult ventricular cell. The lower panel shows the I-V relationship from the same cell. Phenylephrine did not alter the L-type current while Bay K 8644 not only increased the current but also shifted the peak current towards more negative potentials as has been reported previously (Hess *et al.*, 1984; Bean *et al.*, 1986). Two additional cells were pretreated with propranolol (10 μM) and phenylephrine had no effect on the L-type Ca^{2+} channel current (data not shown). The effect of phenylephrine on the L-type current was repeated several times. Fig. V-5. shows results from a group of cells (n=4). These results show that the L-type Ca^{2+} channel current in adult rat ventricular myocytes are not modulated by α_1-adrenoceptor activation.

4. **Discussion**

In these experiments, an inward Ba^{2+} channel current which is sensitive to dihydropyridines was measured in freshly dissociated adult rat ventricular myocytes. The current records showed some inactivation during the 400 ms depolarization. The inactivation shown in Fig. V-2 is not as prominent as in some previous reports and the current amplitude is larger (Ravens *et al.*, 1989). This is due to the fact that 20 mM Ba^{2+} was used as the charge carrier in these experiments while Ca^{2+} was used in their experiments (Ravens *et al.*, 1989). Twenty mM Ba^{2+} was used as the charge carrier in this thesis in order to make a comparison between neonatal and adult rat ventricular myocytes. In adult rat myocytes, the channel activated at -20 mV and the peak amplitude was around +10 mV. According to the electrophysiological and pharmacological properties, the characteristics of this inward Ca^{2+} channel current are similar to the L-type Ca^{2+} channel current described by Bean (1989).

These results show that, in contrast to the results from neonatal rat myocytes, phenylephrine did not affect the L-type current in adult rat ventricular myocytes. The results from these experiments are similar to other studies carried out using cardiac tissue from different species. In adult myocytes, 10 μM phenylephrine or methoxamine had no effect on the Ca^{2+} channel current (Apkon & Nerbonne, 1988; Ravens *et al.*, 1989; Tohse *et al.*, 1990; Terzic *et al.*, 1991). In rabbit atrial and ventricular cells, and guinea-pig and cat myocytes, an effect of phenylephrine on Ca^{2+} channel currents was not obtained (Fedida *et al.*, 1990; Hartman *et al.*, 1988; Hescheler *et al.*, 1988a). In contrast an increase in I_{Ca} was reported in frog myocytes (Alvarez *et al.*, 1987) and bovine Purkinje fibers (Brückner & Scholz, 1984). However, recent studies have demonstrated that the stimulation of α_1-adrenergic receptors in heart results in a decrease in the transient outward K^+ current, which might account for the prolonged action potential and positive inotropic response to α_1-agonists as described in Chapter I. One major reason for the differences between reported observations in I_{Ca} and K^+ current could be related to tissue as well as species differences as suggested by Endoh *et al.* (1991).

Neonatal and adult ventricular myocytes are strikingly different. The α_1-adrenergic receptor density (Nakanishi *et al.*, 1989; Rosen & Robinson, 1990), the different chronotropic responsiveness to α_1-agonists (Kimball *et al.*, 1991), the G protein coupling to α_1-adrenoceptors (Han *et al.*, 1989), the ion channels and some other properties could account for the different ionic basis for the positive inotropic responses induced by α_1-adrenergic agonists. Therefore, it is interesting to study the developmental differences between the neonatal and adult in various species. It would be helpful to establish the ionic basis for stimulation of α_1-adrenoceptors at different stages of development. This can be of importance to the understanding of the pathology of the heart. The altered α_1-adrenergic receptor may have critical importance in the pathogenesis of arrhythmias and may influence myocardial cell viability after an ischaemic insult (Kagiya *et al.*, 1991).

Fig. V-1.

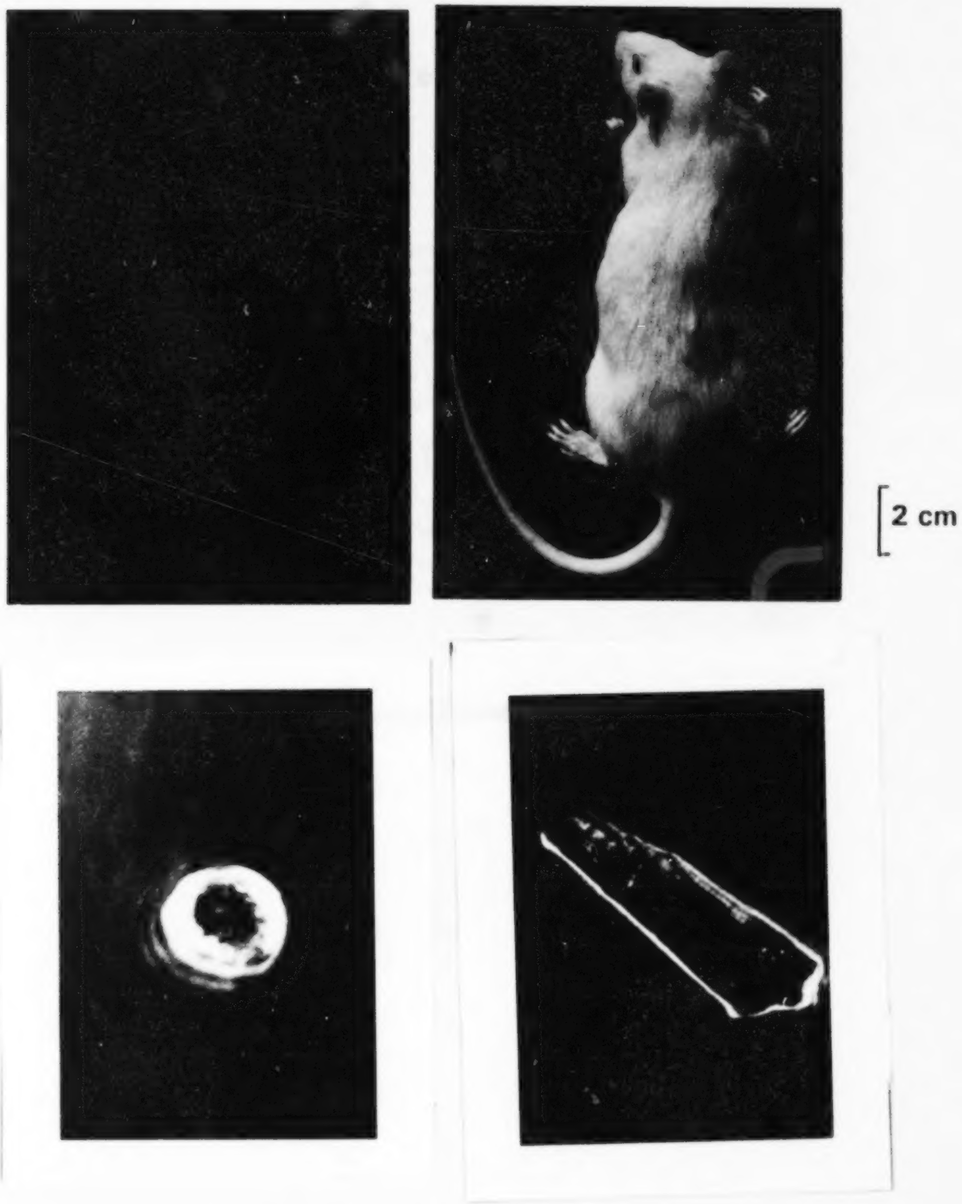


Fig. V-1. The two photographs (top) show neonatal and adult rats used in the experiments. The two photographs (bottom) show ventricular myocytes from neonatal and adult rats. The myocytes from neonatal rat ventricles are round in shape with a diameter of 24 μm. This is in part due to the dissociation procedure. The myocytes from adult rat heart are rod shaped, 84-120 μm in length and 20-24 μm in width.

Fig. V-2.

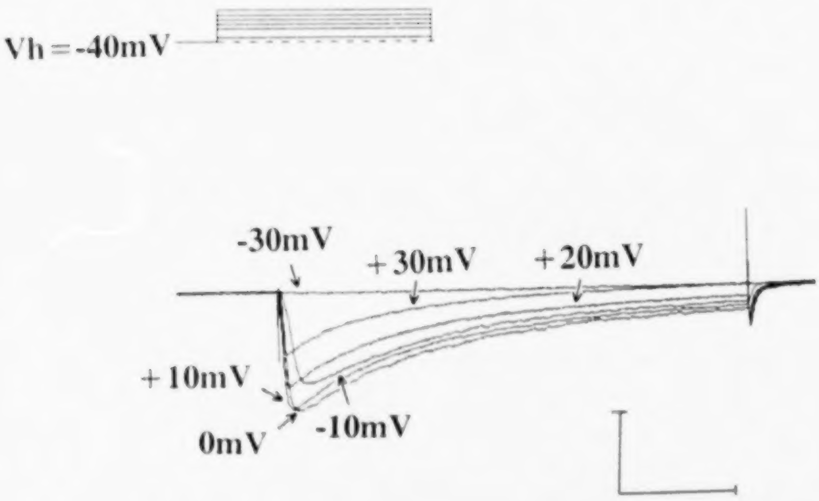


Fig. V-2. Original current records from an adult rat ventricular cell. The L-type Ca^{2+} channel currents were activated by depolarizing the cell to various test potentials from a holding potential of -40 mV. The test potentials are indicated in the figure. Calibration bar: 100 ms and 1 nA.

Fig. V-3.

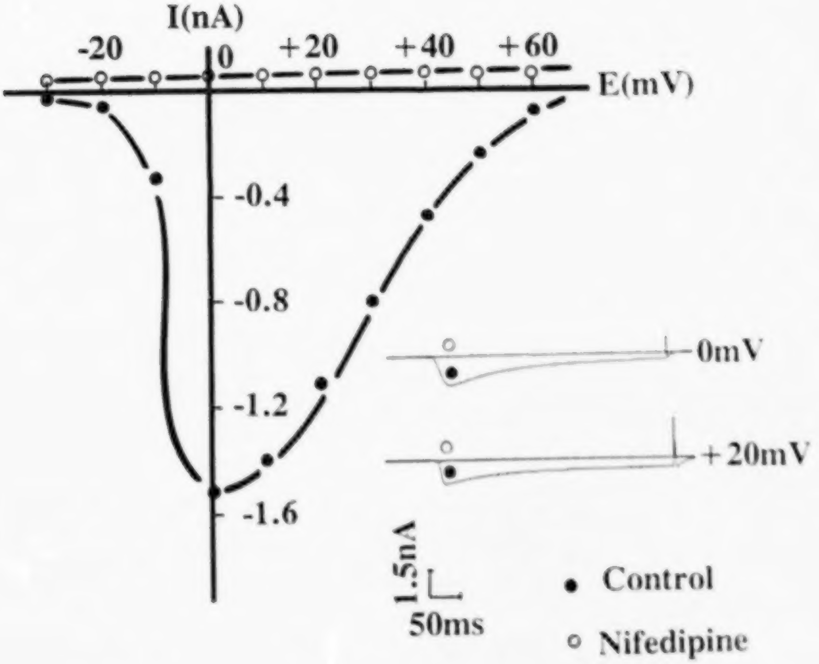


Fig. V-3. Effect of nifedipine (10^{-5} M) on the L-type Ca^{2+} channel current in an adult rat ventricular myocyte. The I-V relationship shows that the current was activated at -20 mV and the peak occurred at approximately 0 mV. Nifedipine completely blocked this inward current. Two original current records from the same cell before and after the addition of nifedipine is also shown in the inset.

Fig. V-4.

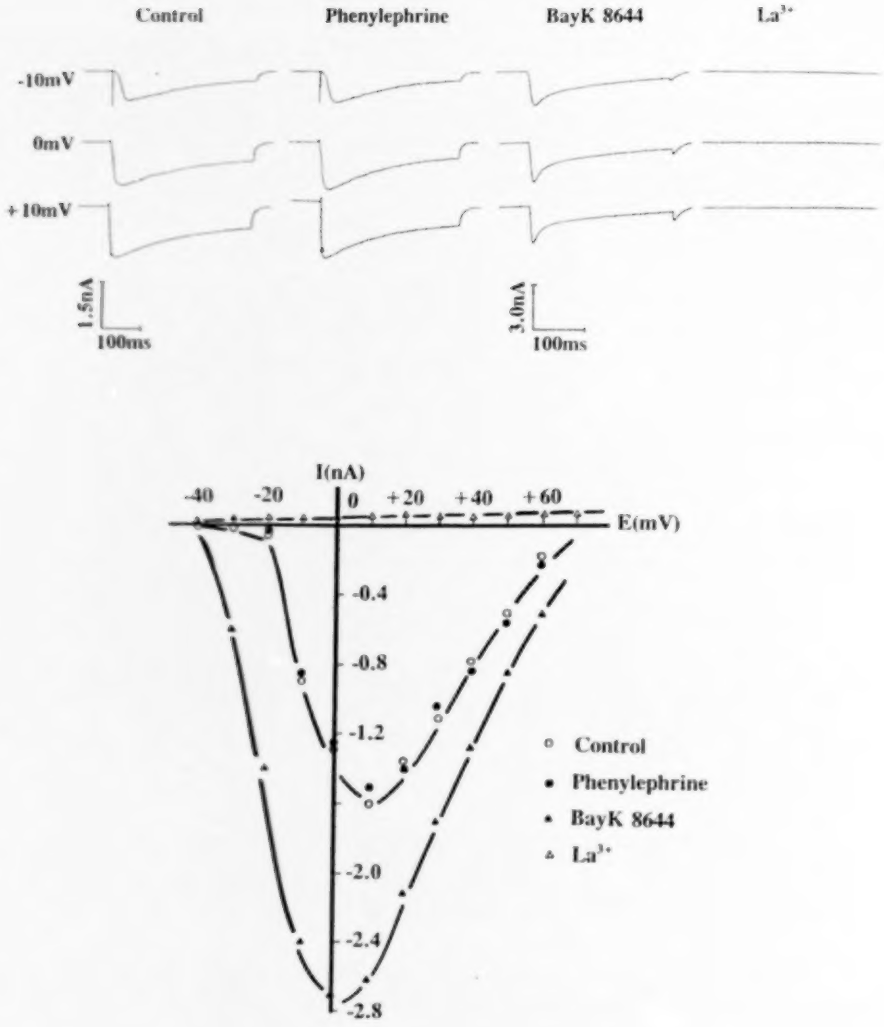


Fig. V-4. Effect of phenylephrine (10^{-5} M) on the L-type current in a single ventricular cell. Upper panel: Original current records (L channel) from one cell. Phenylephrine had no effect on the L-type Ca^{2+} channel current. Bay K 8644 (10^{-6} M) increased the current and La^{3+} (2 mM) completely blocked the current. Note that the calibration bars are identical for the control and phenylephrine experiments, but different for the Bay K 8644 experiments. Lower panel: I-V relationship plotted from the current records shown in the upper panel. Phenylephrine did not affect the L-type channel current. Bay K 8644 not only increased the current amplitude also shifted the peak current towards more negative potentials. La^{3+} completely blocked the L-type current.

Fig. V-5.

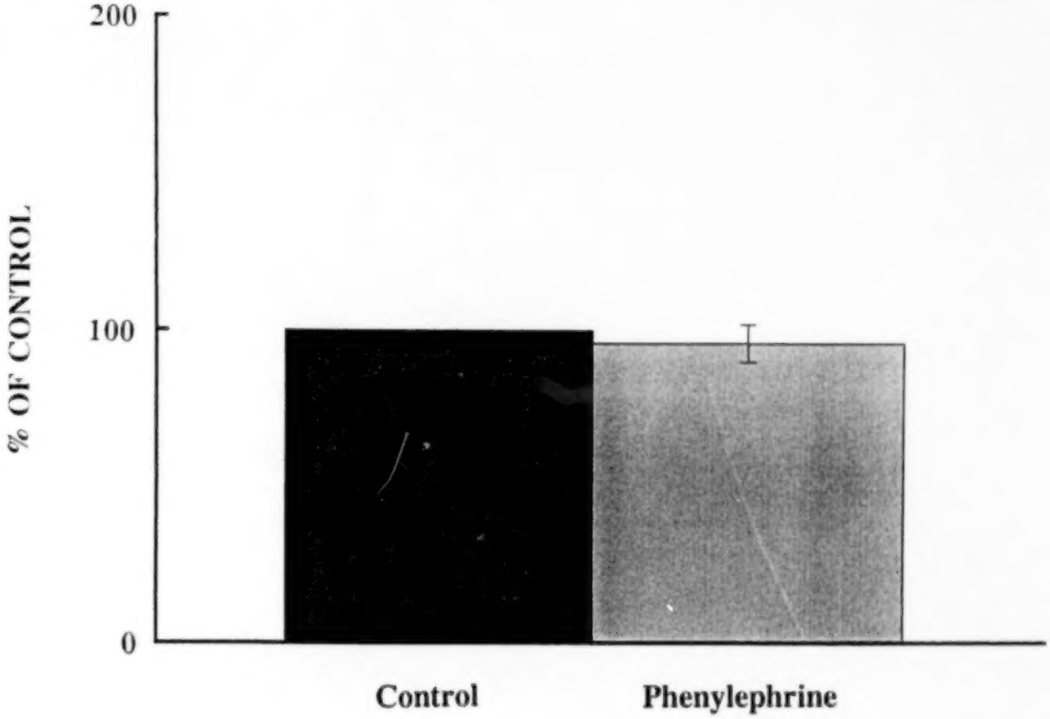


Fig. V-5. Effect of phenylephrine (10^{-5} M) on the L-type current in groups of cells. The effect of phenylephrine was not observed in adult rat ventricular myocytes. Data are expressed as percentage of control, mean ± S.E.M. (n=4).

CHAPTER VI. GENERAL DISCUSSION AND SUMMARY

1. General Discussion

1.1. Modulation of Voltage-Dependent Ca^{2+} Channels

The modulation of time- and voltage-dependent Ca^{2+} channels by neurotransmitters and hormones is critical, since Ca^{2+} influx is unique when compared to that of other ions. In addition to producing an electrical signal (depolarization), Ca^{2+} acts as a cytoplasmic messenger which triggers secretion of neurotransmitters or hormones, contraction of muscle, activation of Ca^{2+}-calmodulin kinase, protein kinase C and numerous proteases. It is widely accepted that G proteins couple receptors to VDCCs and, in most cases, the modulation of VDCCs by neurotransmitters and hormones is mediated by G proteins.

The investigations in this thesis focus on the modulation of voltage-dependent Ca^{2+} channels. Modulation of voltage-dependent Ca^{2+} channels is observed as changes in the voltage-activated current. The changes in whole cell current (increase or decrease) may be initiated by receptor and ligand interaction. The underlying mechanism of modulation involves changes in gating modes and/or phosphorylation. Since many ionic channels are directly or indirectly controlled by ligands, a need for classification is required. The literature concerning this classification is difficult to assess. Hille (1989) separated channels into two classes: voltage-dependent channels and ligand-gated channels. Voltage-dependent channels are channels which are activated by membrane depolarization and are very selective for ions such as Na^+, K^+, Ca^{2+} and Cl^-. The ligand-gated channels require a ligand in order to activate the channel. According to the classification of Hille, ligand-gated channels consist of two basic subtypes. The first subtype is one in which the receptor and ion channel are combined in one molecule. The second subtype of the ligand-gated channel consists of a receptor and a channel which are not combined. Receptors on the cell surface interact with neurotransmitters and hormones. The interaction of receptors and ligands causes the activation of G proteins which may initiate a second messenger cascade. This second messenger cascade or G protein then modulates the channels. This second type of ligand-gated channel is a voltage-dependent channel (Ca^{2+} channel), and should be classified as such. Hence, Brown and Birnbaumer (1988) proposed a third class of ion channels, i.e., G protein-gated channels. The second subtype of the ligand-gated channel in Hille's classification was then considered as this third class of channel (G protein-gated channel). Modulation of these channels is mediated by G proteins. The G protein-gated Ca^{2+} channel does not require G protein activation for channel opening, but membrane depolarization is required (Brown & Birnbaumer, 1990). Although this classification of the G protein-gated Ca^{2+} channel by Brown is helpful in clarifying the classification of ligand and voltage-gated channels, there are still some problems with the identity of these Ca^{2+} channels. Tsien and Tsien's (1990) classification of Ca^{2+} channels is more useful. The voltage-dependent Ca^{2+} channel is the depolarization activated Ca^{2+} channel and this channel may potentially be modulated by neurotransmitters and hormones. The ligand-gated Ca^{2+} channel is the channel which absolutely requires the presence of a ligand for activation, for example, the NMDA- and ATP-gated channels. The study of G protein modulation of VDCC in this thesis deals with this third class of channels proposed by Brown & Birnbaumer (1988). In these experiments, the depolarization of the cell membrane was used to activate voltage-dependent Ca^{2+} channels. A change in current amplitude was observed after application of GTPγS. The results in Chapter IV revealed that the phenylephrine-enhanced Ca^{2+} channel current was sensitive to the DHP (nifedipine). This phenylephrine-enhanced current may be the same as the L-type current activated by depolarization before addition of phenylephrine. The effect of phenylephrine on VDCC was observed to be mediated by a G protein. These experiments show that voltage-dependent Ca^{2+} channels are modulated by G proteins and ligand receptor interaction. The results from this study provide a good example of a modulated voltage-dependent Ca^{2+} channel as described in the classification of Tsien and Tsien (1990).

It is clear that in most cases the modulated Ca^{2+} channels are voltage-dependent channels. However, in recent reviews, many of these modulated ion channels are still categorized as ligand-gated channels. These modulated Ca^{2+} channels are voltage-dependent, but the additional stipulation has to be made that a receptor-mediated pathway may modulate the channel but is not responsible for the primary gating event (Breitwieser, 1991). There are good reasons in many cases to classify modulated ion channels as ligand-gated channels. The classification of channels has focused on channels which are permeant to ions other than Ca^{2+} and it is reasonable to classify many of these channels as ligand-gated channels. In most cases, the ligand only plays a modulatory role. However, the ligand is necessary for the activation of some ligand-gated channels. For example, acetylcholine (ACh) and the G protein are obligatory for opening the muscarinic K^+[ACh] channel. This channel responds to ACh and ACh opens the K^+ channel through the activation of a G protein (G_k) (Codina *et al.*, 1987). This K^+ channel may be another type of channel rather than a voltage-dependent background K^+ channel (Pfaffinger *et al.*, 1985; Brown & Birnbaumer, 1990). However, this is not the case for Ca^{2+} channels and most voltage-dependent ion channels which are modulated by neurotransmitters and hormones. Thus, in future studies and interpretation of data researchers should consider these important points. A reasonable classification of voltage-dependent and ligand-gated channels is necessary to help clarify this area.

1.2. Different Effects of GTPγS on VDCCs in Four Types of Cells

In this thesis, the effect of GTPγS, a G protein activator, on VDCC in four cell types was studied in order to obtain the general scheme of the interaction of G proteins and voltage-dependent Ca^{2+} channels in different tissues. T and L channels were studied using the whole cell version of the patch clamp technique and they were identified by means of electrophysiological characteristics. In the first part of the thesis, the main focus was on the modulatory effect of G proteins on Ca^{2+} channel currents rather than accurate separation of the channel currents. Experiments using four types of cells have clearly demonstrated a different pattern of G protein modulation of VDCCs which is summarized in Table 1. The Ca^{2+} channels in three of the cell types respond to GTPγS and the T channel in N1E-115 cell does not. It is clearly demonstrated that GTPγS has different actions on VDCCs in different tissues. This may be accounted for by several factors.

1.2.1. ***Different G Proteins***

It is generally accepted that different tissues possess distinct G proteins which generate different responses because distinct G proteins couple to different effectors. There are at least 16 different G proteins (Simon *et al.*, 1990). Even in the same cell, there are likely to be several different G proteins present in the membrane. In neuronal tissues, considerable quantities of G_o and G_i are present in the cytoplasmic membrane. These G proteins mediate the inhibitory effects of hormones on Ca^{2+} channel currents (Schultz *et al.*, 1990). One exception which was reported by Scott *et al.* (1988) was that 6 μM caged-GTPγS increased T channel current in DRG neurons. In the results reported in this thesis, no effects of GTPγS were observed on T channel currents in N1E-115 cells. Endocrine tissues contain G proteins, the majority of which are PTX-sensitive, i.e, G_o and G_i, and mediate the inhibitory effect of most hormones. For example, somatostatin (Lewis *et al.*, 1986) and baclofen (Dolphin & Scott, 1987) reduced the Ca^{2+} channel current via PTX-sensitive G proteins. However, PTX-sensitive G proteins have also been reported to mediate the stimulatory effect on Ca^{2+} channel currents by LHRH (Rosenthal *et al.*, 1988a) and angiotensin II (Hescheler *et al.*, 1988b) in endocrine cells. The data in this thesis show that GTPγS has biphasic effects on Ca^{2+} currents in GH_3 cells. GTPγS increased both rapidly and slowly inactivating Ca^{2+} channel current components 2-3 minutes after the initial current and subsequently decreased the currents after

5 minutes. Toxin studies have revealed that a PTX-sensitive G protein is responsible for the initial increase and a CTX-sensitive G protein is responsible for the subsequent decrease in currents. However, more work is required to identify the subtype of G protein. In the case of vascular smooth muscle cells and cardiac myocytes, GTPγS caused an increase in inward Ca^{2+} channel currents.

1.2.2. ***Different Roles of G Proteins***

A cell can contain various G proteins which cause different responses. Alternatively, the same G protein may be coupled to different effectors and produce different responses. In different tissues, the same G protein generated distinct actions on VDCC. For example, in bovine glomerulosa cells, G_i was responsible for the LHRH and angiotensin II-induced increase in VDCC current (Cohen *et al.*, 1988). In DRG neurons, G_i was responsible for the inhibition of VDCC current induced by bradykinin (Ewald *et al.*, 1989). The same G protein could couple to a different Ca^{2+} channel. The G protein could couple to a DHP-sensitive (L-type) Ca^{2+} channel instead of a T-type Ca^{2+} channel. Therefore, the activation of G proteins would result in an increase in the L channel current with no effect on the T current. The organization of G protein-mediated signal transduction is complicated. More studies dealing with specific signal transduction pathways are necessary to sort out these complexities. This study of the effect of GTPγS on VDCCs in different cell types under the same experimental conditions provides some useful information. After enough detailed information is obtained, the overall scheme of G protein modulation of VDCC could be established.

1.3. G Protein Mediation of a Specific Pathway in Cardiac Myocytes

Many studies have shown that VDCCs can be modulated by the activation of receptors via G proteins. In cardiac myocytes, Ca^{2+} channel modulation is of the utmost importance. The β-adrenergic activation-mediated modulation of VDCC in cardiac cells has been well documented. Basically, there are two pathways for β-adrenergic modulation of VDCC (Trautwein & Hescheler, 1990). 1) β-Adrenoceptor---G_s---AC---cAMP---PKA----VDCC and 2) β-Adrenoceptor---G_s---VDCC. The responses to α_1-adrenoceptor activation in the heart have been well characterized. Both of these adrenergic receptors can induce positive inotropic and chronotropic responses in heart. However, unlike the β-adrenoceptor, the mechanism of the activation of the α_1-adrenoceptor induced-positive inotropic response has not been clearly delineated.

It has been reported that the stimulation of α_1-adrenoceptors in adult animal heart resulted in the depression of transient outward K^+ current (Fedida *et al.*, 1991). The mechanism in neonatal rat heart as described in this thesis has been shown to be different from that in the adult rat heart. For example, the transient outward current in neonatal ventricular cells is small when compared that of the adult rat (Kilborn *et al.*, 1990). The α_1-adrenergic agonist phenylephrine was used to demonstrate that the stimulation of α_1-adrenoceptors in the neonatal rat heart induced an increase in L-type Ca^{2+} channel current. These results are summarized in Table 2. This is the first report to show that the α_1-agonist phenylephrine increased L-type Ca^{2+} channel current in neonatal rat ventricular cells.

It has been described in Chapter III that intracellular application of GTPγS increased the L-type Ca^{2+} channel currents in neonatal rat ventricular cells. GTPγS (500 μM) not only increased the current amplitude but also shifted the peak current of the I-V relationship towards more negative potentials (5-10 mV). Phenylephrine not only increased the L-type current, but also shifted the peak current towards more negative potentials. These results were similar to those obtained with GTPγS. The effect of phenylephrine on the L-type Ca^{2+} channel current was greater in the negative potential range and a similar effect of GTPγS on Ca^{2+} channel current was observed. The steady-state activation and inactivation experiments showed similarity between GTPγS and phenylephrine. Both of them shifted the steady-state activation and inactivation towards more negative potentials. Therefore, GTPγS could mimic the effect of phenylephrine on the L-type Ca^{2+} channel current. This supports the conclusion that a G protein is required in the α_1-adrenergic agonist phenylephrine-induced increase in Ca^{2+} current in neonatal rat ventricular cells. In addition, GTPγS enhanced the phenylephrine effect and GDPβS blocked the phenylephrine-induced Ca^{2+} channel current increase. These results are summarized in Table 3.

The G protein which is coupled to the α_1-adrenoceptor as described in this thesis is a PTX- and CTX-insensitive. This is consistent with other reports based on neonatal rat ventricular cells (Bilezikian *et al.*, 1988). It has been reported recently that a new class of G proteins, G_q, which is insensitive to PTX, has the ability to hydrolyze PIP_2 into IP_3 and DG (Pang & Sternweis, 1990; Smrcka *et al.*, 1991; Taylor *et al.*, 1991). Whether G_q is the G protein that couples to α_1-adrenoceptors in neonatal rat heart is uncertain. In order to identify the specific G protein, more studies using biochemical and molecular biological methods must be conducted.

After the G protein involvement was established, the next stage was to describe the second messenger pathway which activates the L-type Ca^{2+} channel. The results from Chapter IV clearly demonstrate that the effect of phenylephrine on the L-type Ca^{2+} channel current was mediated by PKC and not by PKA. These results were supported by previous reports (Buxton & Brunton, 1986; Steinberg *et al.*, 1987, 1989). The evidence that PKA did not mediate the α_1-adrenergic agonist-induced increase in Ca^{2+} current is as follows. 1) S_p-cAMPs, a PKA activator, did not affect the phenylephrine-induced increase in Ca^{2+} current. 2) R_p-cAMPs, a PKA inhibitor, did not block the effect of phenylephrine on the L-type Ca^{2+} current. 3) The G protein involved is not cholera toxin-sensitive and, hence, the possibility that G_s mediates this effect is unlikely. However, the PKC pathway was clearly shown to be involved in the phenylephrine-induced effect on Ca^{2+} channel currents. Evidence for this is as follows. 1) PKC activators, PMA and OAG, mimicked the effect of phenylephrine on L-type Ca^{2+} channel current. 2) The PKC inhibitor, staurosporine, blocked the effect of phenylephrine on L current. 3) Pretreatment of cells with a high concentration of PMA to deplete endogenous PKC abolished the effect of phenylephrine on the L channel current. 4) The G protein involved in this response is a PTX- and CTX-insensitive G protein. It has been shown that a PTX-insensitive G protein (G_q) is involved in the PIP_2-PKC pathway (Smrcka *et al.*, 1990; Wange *et al.*, 1991) and, hence, as stated previously, G_q might be responsible for the phenylephrine response. The linkage between PKC and G protein in this α_1-adrenegic activation-induced increase in L-type Ca^{2+} channel current was proposed in this thesis as PLC pathway. However, other mechanisms by which PKC may be activated (such as arachidonic acid, PIP_2 and elevated $[Ca^{2+}]_i$) cannot be excluded.

The pathway of the α_1-adrenoceptor activation in neonatal rat heart was mapped out using several agents, i.e, α_1-adrenoceptor---a PTX- and CTX-insensitive G protein---PLC---DG---PKC---L-type Ca^{2+} channel. Phenylephrine binding to α_1-adrenoceptors activated a PTX- and CTX-insensitive G protein which caused an intracellular cascade, eventually leading to phosphorylation of the DHP-sensitive Ca^{2+} channel proteins. The description of this pathway improves our understanding of the mechanism underlying α_1-adrenergic stimulation in neonatal rat heart. It may also contribute to the knowledge of developmental changes and the pathology of the heart. Although it has been reported that there is a PTX-insensitive G protein coupling the α_1-adrenoceptor to PLC which hydrolyzes PIP_2 to IP_3 and DG in heart (Steinberg *et al.*, 1987), this is the first report to relate this G protein to the intracellular pathway and the Ca^{2+} channel. Although many other G proteins have been shown to be involved in the modulation of VDCCs, this report shows clearly that a PTX-insensitive G protein mediated the phenylephrine-induced increase in the L-type Ca^{2+} channels in neonatal rat myocytes. The study of this mechanism could account for the positive inotropic response induced by α_1-adrenergic stimulation in the heart. The modulation of VDCCs is a complicated and subtle process which is critical to cellular activities. It has been demonstrated that PKC activators increased Ca^{2+} channel currents in neonatal rat ventricular cells (Dosemeci *et al.*, 1988; Lacerda *et al.*, 1988). This was confirmed in this thesis and suggests that PKC modulation of VDCC may be physiologically important. The description of this pathway extends our knowledge not only of the mechanism underlying the stimulation of α_1-adrenoceptors, but also of the modulatory process.

1.4. Comparison of Neonatal and Adult Rat Myocytes

This is the first report to compare the effect of phenylephrine on the L-type Ca^{2+} channel currents in neonatal and adult rat heart. The response to the α_1-adrenergic agonist, phenylephrine, is different in neonatal and adult ventricular myocytes with regard to L-type Ca^{2+} channel current as shown in Chapters IV and V. The reasons for this difference may be as follows. 1) There are morphological differences between the two types of cells. Therefore, the structural differences, for example, a spanning protein as described in Chapter I (3.3 and 3.4) may be responsible for differences in mechanisms. 2) The α_1-adrenergic receptor density was higher in neonatal rat myocytes than that in adult rat myocytes (Karliner *et al.*, 1985). 3) In neonatal rat myocytes, a PTX-insensitive G protein has been reported to couple to α_1-adrenoceptors (Steinberg *et al.*, 1985) which was similar to the results shown in chapter IV. The adult rat myocytes had both PTX-insensitive and PTX-sensitive G proteins. The PTX-insensitive G protein was responsible for the negative chronotropic response to the stimulation of the α_1-adrenoceptors while a PTX-sensitive G protein was responsible for the positive chronotropic response due to the stimulation of the α_1-adrenoceptors (Han *et al.*, 1989). In neonatal rat ventricular myocytes, only a PTX-insensitive G protein was present and was responsible for positive chronotropism (Han *et al.*, 1989). 4) The mechanisms of positive inotropism induced by stimulation of α_1-adrenoceptors were reported due to suppression of I_t (transient outward

K^+ current) in adult myocytes (Fedida *et al.*, 1991). However, the current density of I_t in day 1 to day 3 neonatal rat ventricular cells is low and increased in density with development (Kilborn & Fedida, 1990). Therefore, I_t is of little functional importance in the early stage of development of the newborn rat heart. In embryonic rat heart cells, it has been reported that the first channel to be expressed is the Ca^{2+} channel (Bernard, 1975). In addition, the neonatal ventricular action potential is longer, with a more defined plateau phase, than that in adult rat (Chapter 4). This suggests that a functional inward Ca^{2+} current is present. Hence, it is likely that the α_1-adrenoceptors are coupled to Ca^{2+} channels through an intracellular cascade at the early stage of development and then shift to the transient outward K^+ channels in the mature state. Therefore, an increase in Ca^{2+} channel current is responsible for the prolonged action potential in neonatal rat heart whereas a decrease in K^+ channel current is responsible for the action potential change in adult rat heart (Kilborn & Fedida, 1990; Fedida *et al.*, 1990).

1.5. Approaches for the Study of G Protein Modulation of VDCC

The intracellular application of GTPγS results in the activation of all G proteins. The results would thus be the summation of all activated G proteins which couple to the specific effectors, i.e., Ca^{2+} channels in a certain cell. It is conceivable that GTPγS could increase, decrease or have no effect on Ca^{2+} channel currents in different tissues. As a G protein activator, the use of GTPγS intracellularly gives an indication that a G protein is involved in the modulation of Ca^{2+} channels. The unequivocal determination of the nature of the G protein involvement requires a variety of experimental approaches.

The commonly used methods in electrophysiology include the following. 1) A direct receptor-independent activation of the G protein by hydrolysis-resistant GTP analogues, such as GTPγS which was used in this thesis. 2) Modification of the G protein by pertussis toxin or cholera toxin which was also demonstrated in some of the experiments. 3) Inhibition of the receptor-mediated effects by GDPβS. GDPβS was used in this thesis to block the phenylephrine-enhanced L channel current in myocytes. 4) Use of the specific antibodies to the G protein subtype to interfere with the signal transduction process and the purified G protein subunit to restore the reactions. This provides conclusive evidence of the involvement of G proteins and the identification of the specific G protein. Although all the approaches listed above, i.e. specific antibodies, were not used in this thesis, experiments using GTPγS, CTX and PTX, and GDPβS provide strong evidence that G proteins are involved in the modulation of Ca^{2+} channels. G proteins are known to couple to the receptors and effectors (Brown, 1991). Once a receptor is activated, a specific G protein which couples to that receptor will be activated followed by the effector activation. For example, phenylephrine, an α_1-adrenergic agonist, binds to α_1-receptors in myocytes and this leads to activation of a specific G protein. This G protein, in turn, activates Ca^{2+} channels directly and/or indirectly through second messengers. Therefore, with the help of an agonist, the complete mechanism which includes the specific G protein and second messenger cascade can be determined. This constitutes the second part of the thesis.

The experiments were all performed using the whole cell version of the patch clamp technique. This technique allows the recording of currents generated by many ion channels in the cell membrane which open in response to changes in voltage. It also allows large molecules to be introduced into the cell. GTP and GDP analogues and their photolabile caged precursors, or other compounds which interfere with the intracellular reactions, can be applied to the cell's interior. In the same experiment, other chemical compounds, such as neurotransmitters, hormones and drugs can be applied to the cell's exterior. The experiments were conducted in an intact cell which contains the entire signal transduction pathway. This technique provides adequate conditions in which to study the signal transduction process.

It is difficult to extrapolate results from patch clamp experiments in which the cells are dialysed to normal physiological conditions. The experiments described in this thesis were carried out under conditions in which a high concentration of Ba^{2+} replaced Ca^{2+} as the charge carrier, and the influence of all other ions was excluded. The intracellular Ca^{2+} concentration was clamped at a pCa of 7-8 using a Ca^{2+} EGTA buffered solution. This internal concentration of Ca^{2+} should not affect the activities of intracellular Ca^{2+}-dependent enzymes, but other important components, i.e. calmodulin, may be washed out. It is surprising that the whole tissue was less responsive to phenylephrine than were the dialysed cells. A possible explanation is that, in the whole tissue experiments, other mechanisms may counteract the effect of phenylephrine on the L-type Ca^{2+} channel, whereas in dialysed cells the influence of other ions was excluded.

It has been shown that in DRG neurons, the inhibitory action of GTPγS was progressive with time. Solutions containing 100 μM GTPγS required 3-4 minutes to reach maximal inhibition in cells of 30 μm diameter (Pollo *et al.*, 1991). In this thesis, it was also observed that the intracellular application of GTPγS caused an increase in Ca^{2+} channel currents in 2-3 minutes. The time in these experiments for obtaining the effect was similar to previous reports (Dolphin *et al.*, 1989; Pollo *et al.*, 1991). It has been reported that a complete replacement of the intracellular ionic composition required 5 minutes (Doroshenko *et al.*, 1982). In this thesis, a period of 10 minutes after rupture of the membrane was used to allow a complete dialysis of the intracellular composition before addition of the drugs to the bath solution, as indicated in the Results section.

1.6. Modulation of VDCC by G Protein Directly or Indirectly

It has been demonstrated that G proteins can act directly on Ca^{2+} channels (Yatani *et al.*, 1987b; Pelzer *et al.*, 1990) or indirectly through second messengers (Trautwein & Hescheler, 1990). Purified $G_{s\alpha}$ was shown to activate the DHP-sensitive Ca^{2+} channels which were incorporated into the lipid bilayer. This activation of Ca^{2+} channels was independent of PKA, PKC and ATP (Yatani *et al.*, 1987a, 1988a). β-Adrenergic agonists increased Ca^{2+} channel current through the direct G protein pathway (Yatani & Brown, 1989). β-adrenergic agonists also increased Ca^{2+} channel currents through G_s and intracellular cAMP-dependent phosphorylation (Nargeot *et al.*, 1983; Yatani & Brown, 1989). This was observed by applying rapid jumps of the β-adrenergic agonist isoproterenol (ISO) to cardiac atrial cells using the concentration clamp method (Akaike *et al.*, 1986). The increase in Ca^{2+} channel currents was biphasic with a time constant of 150 ms for the fast direct G protein pathway and 36 seconds for the slow indirect pathway (Yatani & Brown, 1989).

It was shown in this thesis that phenylephrine increased L-type Ca^{2+} channel current through an indirect pathway, which is similar to the action of ISO. However, a much slower phase of increase in Ca^{2+} channel current was observed in these experiments. Ca^{2+} current started to increase 5 minutes after addition of phenylephrine. This may in part be attributed to the diffusion of the drug. Although PKC inhibitors completely blocked the phenylephrine-enhanced Ca^{2+} channel current, a direct G protein pathway from α_1-adrenoceptor to L-type Ca^{2+} channel current cannot be excluded. This direct G protein pathway could not be measured in these experiments because the time resolution in the experimental procedure was inadequate. Other techniques, such as single isolated patch recording, purified channel protein reconstitution and concentration jump are required to determine the possibility of this direct G protein pathway.

1.7. G Protein-Gated Ca^{2+} Channels in the Presence and Absence of the Ligand

Postsynaptic membrane noise due to the release of neurotransmitters produces stochastic openings and closings of ligand-gated ion channels. However, this may not be the only mechanism by which noise is produced. In postsynaptic atrial muscle cells acetylcholine activates K^+[ACh] via G_k (G_{i-3} or G_{i-2}) (Breitwieser & Szabo, 1985; Codina *et al.*, 1987; Yatani *et al.*, 1988b). However, atrial K^+[ACh] channels have been reported to open spontaneously in the absence of an applied agonist (Soejima & Noma, 1984; Logothetis *et al.*, 1987). This phenomenon is not limited to excitable cells. Spontaneous membrane noise also occurs in G protein-gated K^+ channels in non-excitable membranes and may apply to other ion channels (Okabe *et al.*, 1991). Therefore, under physiological conditions, not only can the activation of a receptor initiate the signal transduction process in the G protein-receptor coupling system, but also the agonist-free, empty receptor may initiate the signal transduction process. This may be of physiological significance. This thesis deals with two major objectives, the modulation of VDCC by direct activation of G protein (agonist-free) and the modulation of VDCC by a G protein-receptor coupling system. Both of these situations may exist under physiological conditions. These studies are, therefore, important for our understanding of modulation of VDCC by various mechanisms, in which G protein modulation of VDCC may be the most important.

2. Summary

2.1. GTPγS Affected Ca^{2+} Channel Currents in a Different Pattern in Four Cell Types

2.1.1. *GH_3 Cells*

The intracellular application of GTPγS increased both slowly and rapidly inactivating Ca^{2+} channel components in the first 2-3 minutes. It decreased them after 5 minutes. A PTX-sensitive G protein was responsible for the initial activation of the Ca^{2+} channel and a CTX-sensitive G protein was responsible for the subsequent inhibition of the Ca^{2+} channel.

2.1.2. *N1E-115 Cells*

GTPγS had no effect on T-type Ca^{2+} channel currents.

2.1.3. *VSMC Cells*

GTPγS increased L-type and total inward (both T and L) Ca^{2+} channel currents. GTPγS shifted the peak of the I-V relationship of Ca^{2+} currents towards more negative potentials. No subsequent inhibition was observed.

2.1.4. *Neonatal Rat Ventricular Myocytes*

GTPγS not only increased both T- and L-types Ca^{2+} channel currents, but also shifted the peak of the I-V relationship towards more negative potentials. GTPγS increased Ca^{2+} current and this increase was concentration-dependent. The intracellular application of GTPγS shifted the steady-state activation and inactivation towards more negative potentials. The increase in Ca^{2+} currents by GTPγS was confirmed using flash photolysis and caged-GTPγS. As compared with data from GH_3 cells, no inhibition was observed.

2.2. **Phenylephrine Increased the L-Type Ca^{2+} Channel Current Via a PTX- and CTX-Insensitive G Protein and PKC Pathway in Neonatal Rat Ventricular Cells**

2.2.1. *Phenylephrine Increased Ventricular (Neonatal Rat) Tension*

This increase was concentration-dependent. Phenylephrine also prolonged the plateau phase of action potentials in single ventricular myocytes.

2.2.2. *Phenylephrine Increased the L-Type Ca^{2+} Channel Current*

This increase was specifically mediated by the α_1-adrenoceptor. This effect was concentration-dependent and slow. The enhanced current was dihydropyridine sensitive. Phenylephrine shifted the steady-state activation and inactivation curve towards more negative potentials. Phenylephrine did not affect the T-type Ca^{2+} channel current in the same preparations.

2.2.3. *A PTX- and CTX-Insensitive G Protein Pathway Mediated the Phenylephrine-Induced Increase in L-Type Ca^{2+} Channel Current*

GTPγS enhanced the phenylephrine effect and GDPβS blocked the phenylephrine effect on the Ca^{2+} channel current. Pretreatment of myocytes with pertussis toxin and cholera toxin did not abolish the phenylephrine effect on the Ca^{2+} channel current.

2.2.4. *PKC Mediated the Phenylephrine-Induced Increase in L-Type Ca^{2+} Channel Current*

Protein kinase C activators, PMA and OAG, mimicked the phenylephrine effect and PKC inhibitors, staurosporine and prolonged treatment of cells with high concentration of PMA, blocked the phenylephrine effect on Ca^{2+} channel current. The PKA activator and inhibitor, S_p-cAMPs and R_p-cAMPs, respectively, did not affect the phenylephrine-induced modulation of Ca^{2+} channel current.

2.3. **Phenylephrine Did Not Affect the L-Type Ca^{2+} Channel Current in Adult**

Rat Ventricular Myocytes

The L-type Ca^{2+} channel current in adult myocytes did not respond to phenylephrine but responded to Bay K 8644 and nifedipine. This L channel current could be completely blocked by La^{3+}.

3. Significance

1. This is the first report to show that a PTX- and CTX-insensitive G protein is involved in the modulation of the L-type Ca^{2+} channel current in neonatal rat heart.
2. This thesis describes for the first time the α_1-adrenoceptor pathway (mechanism) of G protein coupling of the receptor to the effector in neonatal rat heart. The stimulation of the α_1-adrenoceptor results in the activation of a PTX- and CTX-insensitive G protein. From the literature and this work, it is possible that the α_1 subunit of the G protein dissociates from the $\beta\gamma$ subunits and activates PLC which hydrolyzes PIP_2 into IP_3 and DG. DG then activates PKC which, in turn, phosphorylates L-type Ca^{2+} channel proteins and activates them. This pathway differs from the well established β-adrenoceptor pathway (β-adrenoceptor---G_s---PKA---DHP-sensitive Ca^{2+} channel). It is further shown that a PTX- and CTX-insensitive G protein couples to an α_1-adrenoceptor and L-type Ca^{2+} channels through an intracellular PKC pathway. This provides the ionic basis and mechanism for the α_1-adrenergic inotropic response in the neonatal rat heart.

3. The effect of α_1-adrenergic stimulation was compared in the neonatal and adult rat heart. Phenylephrine did not affect the L channel current in adult ventricular myocytes. The differences in the response to phenylephrine between neonatal and adult rat myocytes may be important in heart development and pathology.

4. This is the first known comparison, under similar experimental conditions, of the effect of GTPγS on Ca^{2+} channel currents in four types of cells. The complicated and subtle G protein modulation of VDCC was explored at the level of these four different subtypes.

Table 1. G Protein Modulation of Voltage-Dependent Ca^{2+} Channels in Four Cell Types

	GH_3 cell	N1E-115 cell	VSMC	Ventricular cell (neonatal)
Ca^{2+} channels	rapidly and slowly inactivating components	T	L and T	L and T
GTPγS	↑ and then ↓	-	↑	↑ dose-dependent
PTX-pretreatment	abolishes the initial increase	-	-	-
CTX-pretreatment	abolishes the delayed decrease	-	-	-
G proteins	PTX-sensitive CTX-sensitive	-	not CTX-sensitive[1]	G_s[2], $G_{(?)}$

1) Zeng, Benishin and Pang (1989) J. Pharmacol. Exp. Ther. 250(1): 343.
2) Yatani *et al.* (1987) Science 238: 1288.

The Ca^{2+} channels and the effect of GTPγS on Ca^{2+} channel currents in four cell types are compared. In GH_3 cells, there are two components of inward current (a rapidly and a slowly inactivating component). GTPγS initially increased both components and subsequently decreased them. A PTX-sensitive G protein may be responsible for the initial increase and a CTX-sensitive G protein may be involved in the subsequent decrease. In N1E-115 cells, only T channel current was observed. GTPγS did not significantly alter the T type Ca^{2+} channel current. In VSMC and neonatal rat ventricular cells, there are two types Ca^{2+} channel currents. GTPγS increased the inward Ca^{2+} channel currents in these two types of cell. The G proteins are not CTX-sensitive G proteins in VSMC. G_s or other G proteins may be responsible for the increase in Ca^{2+} currents in neonatal rat ventricular cells.

Table 2. The Effects of Phenylephrine on L-Type Calcium Channel Currents in neonatal rat ventricular cells

	Phenylephrine	
Tension	dose-dependent ↑	
Action potential	↑ in plateau phase	
L-type calcium channel current	↑	dose-dependent
		shifts the peak of I-V to negative potential
		slow
		not reversible
		DHP-sensitive
		shifts steady-state activation and inactivation
T-type calcium channel current	-	

↑ increase
- no effect

Table 3. Summary of G Protein Involvement in the Stimulation of α_1-Adrenoceptor-Induced L-Type Calcium Channel Activation

	L-type current	
GTPγS	↑	dose-dependent
		shifts the peak of I-V
		shifts the steady state activation and inactivation
GTPγS + phenylephrine	enhancement	
GDPβS + phenylephrine	-	
pertussis toxin + phenylephrine	↑	
cholera toxin + phenylephrine	↑	
phenylephrine	↑	

↑	increase
-	no effect

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